Marine microbial interactions: who secretes what for which purpose

By

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Table of Contents

List of Figures

List of Tables

List of abbreviations

α - alpha

AAnPs – Aerobic anaerobic photosynthesis

ABC – ATP-binding cassette

AHL – N-Acyl homoserine lactone

ASW – Artificial sea water

ATP – Adenosine triphosphate

ATPase – Adenosine triphosphatase

C - Carbon

CAZymes – Carbohydrate-active enzymes

CDS – Coding domain sequences

CFB – Cytophaga-Flavobacteria-Bacteroidetes

CFU – Colony forming units

Cl - Chlorine

cm – centimeter

CO2 – Carbon dioxide

DIP – Dissolved inorganic phosphorus

DMS - Dimethylsulfide

DMSP – Dimethylsulfoniopropionate

DNA – Deoxyribonucleic acid

DOM – Dissolved organic matter

DOP – Dissolved organic phosphorus

e.g. – *exempli gratia*

F – Forward (for describing primers)

Fe - Iron

g – grams

× g – times gravity

GC – guanine-cytosine

g/L – grams per litre

GTA – Genetic transfer agent

Gm – Gentamicin

h – hr(s)

Hcl – Hydrogen chloride

HMW – High molecular weight

i.e. – *id est*

Kb – Kilo base

KDa – Kilo Dalton

Kn – Kanamycin

LB – Lysogeny broth

LDOM – Labile dissolved organic matter

LDS – Lithium dodecyl sulfate

LMW – Low molecular weight

m – meter (for describing distance)

MB – Marine broth

mb – mega base

min – minute

ml – millilitre

mM – millimolar

mm – millimetre

MMA – Monomethyl amine

MMM – Minimal marine media

MOPS – 3-(N-morpholino) propanesulfonic acid

N – Nitrogen

NA – Not applicable

Na – Sodium

NANA – N-acetyl neuramic acid

NCBI – National Centre for Biotechnology Information

$(NH_4)_2SO_4$ – Ammonium sulphate

Ni - Nickel

NO3 – Nitrate

O^{2-} - Superoxide

^oC – Degree Celsius

OMZ – Marine oxygen minimum zones

P – Phosphorus

p – probability value

PAGE – polyacrylamide gel electrophoresis

PCA – Principal component analysis

Pho – Alkaline phosphatase

POM – Particulate organic matter

PUL – Polysaccharide utilization loci

PCR – Polymerase chain reaction

q – False discovery rate

R – Reverse (for describing primers)

RDOM – Refractory dissolved organic matter

ROS – Reactive oxygen species

rRNA – Ribosomal ribonucleic acid

rpm – revolutions per minute

RTX – Repeats-in-Toxin

s – second(s)

S – Sulphur

SDOM – Semi-labile dissolved organic matter

SDS – Sodium dodecyl sulphate

Sus – Starch utilization system

SW – Sea Water

T - Time

TRAP – Tripartite ATP-independent periplasmic transporter

Tris – Tris (hydroxymethyl) aminomethane

µg/ml - microgram per millilitre

µl - microlitre

µM - micromolar

W/V – Weight/Volume

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I would also like to dedicate my thesis to my parents, my in-laws, to my kids who never disturbed me while working from home such as during paper reading and writing thesis, and especially to my husband who supported me at every step and believed at me.

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

– Chapter 4, the experimental set up and the samples were processed by MSc student Natacha Chenevoy.

– Chapter 5, some mutants were provided by other people as mentioned in chapter 5 (Section 5.2.1).

Abstract

This project has allowed to gain new insights on the interactions that occur between marine phototrophs and heterotrophs, which are vital in maintaining the nutrient balance in these environments. Marine phototrophs are almost exclusively dedicated to primary production and carbon fixation processes, and release large amounts of dissolved organic matter (DOM) that sustains the entire marine food web. This DOM is mainly re-mineralised by heterotrophic microorganisms. An important fraction of this DOM is in the form of large biopolymers and, hence, heterotrophs need to secrete hydrolytic enzymes to degrade the DOM outside the cell as microbial membrane transport systems only operate with small molecules. The overarching aim of this project was to explore how marine micro-organisms hydrolyse primary produced biopolymers and to highlight the molecular mechanisms that drive these interactions. Special attention was given to the *Synechococcus* – Roseobacter co-culture system and to the full characterization of detected hydrolytic-like enzymes. For this, a detailed exoproteomic time-course analysis of a 100-day co-culture was generated between the model marine picocyanobacterium *Synechococcus* sp. WH7803 and the Roseobacter strain *Ruegeria pomeroyi* DSS-3, both in nutrient-enriched and natural oligotrophic seawater (Chapter 3). The proteomic data showed a transition between the initial growth phase and stable-state phase that, in the case of the heterotroph, was caused by a switch in motility attributed to organic matter availability. The phototroph adapted to seawater oligotrophy by reducing its selective leakiness, increasing the acquisition of essential nutrients and secreting conserved proteins of unknown function. A surprisingly high abundance of extracellular superoxide dismutase produced by *Synechococcus* and a dynamic secretion of potential hydrolytic enzyme candidates were reported that are used by the heterotroph to cleave organic groups and hydrolase polymeric organic matter produced by the cyanobacterium. Key secreted hydrolytic enzymes and interaction proteins that were detected in the exoproteome of *R. pomeroyi* during the time course experiment were knocked out for further investigation to elucidate their role during phototroph-heterotroph interactions (Chapter 5), although further work is required for full characterisation. For example, to further characterise the pectate hydrolase mutant, the

exoproteome of pectate hydrolase mutant in the presence of different polysaccharides can be analysed or the pectate hydrolase mutant can be overexpressed in *E. coli*. The hydrolytic enzymes can be tested on eco or biolog plates. The exoproteomes of 16 different heterotrophic bacteria were also analysed in the presence of the phototroph *Synechococcus* sp. WH7803 (Chapter 4). The data obtained from this analysis indicated a distinct life strategy of each organism to acquire different sources of DOM and, more interestingly, a clear shift in the presence of the phototroph was seen when they were given a more amenable source of carbon and energy. Special attention was given to the different hydrolytic enzymes reported in the exoproteome of heterotrophs and that were more abundantly detected in the co-culture with *Synechococcus*. This project reflects that phototroph-heterotroph interactions are based on nutrient recycling. The data presented in this thesis will become a reference for understanding the molecular processes underpinning marine phototroph-heterotroph interactions and for future characterisation of key marine hydrolytic enzymes.

Chapter 1

Introduction

1.1 Importance of oceans

The ocean is the Earth's largest biome covering 70% of the world's surface. Marine systems play a major role in global climate regulation, not only due to their ability to store and transport heat, but also because of the constant atmosphere—ocean exchange of CO_2. The ocean plays a very important role in the evolution of atmospheric CO_2, buffering the excess of anthropogenic CO_2 emissions caused by the burning of fossil fuels, deforestation and other activities. Hence, the ocean helps to reduce the rate of climate change. The oceans are also major drivers of nitrogen, phosphorus and sulphur cycles that affects the biogeochemical balance on earth (Bopp et al., 2007). The most common product of degradation of any N-containing compound is ammonium (Solomon et al., 2010), which is rapidly recycled in the water column (Zehr and Kudela, 2011). Ammonium is the preferred N-form of dissolved inorganic nitrogen used by primary producers (Christie-Oleza et al., 2017). Some planktonic species, such as bacteria uses urea as another preferred source of N (Berman and Bronk, 2003, Solomon et al., 2010). Most of the phosphorus in the ocean is in an organic form (dissolved organic phosphorous, DOP; White and Dyhrman, 2013). The P within DOP is cleaved off by alkaline phosphatases and, hence, microbes that contains these enzymes can degrade DOP to obtain phosphorus. Heterotrophic bacteria sometimes also decompose DOP to obtain carbon and nitrogen by leaving behind the remineralised dissolved inorganic phosphorus (White and Dyhrman, 2013). At the surface of the ocean, phototrophs uptake sulfate incorporating it into organic compounds begining the sulphur cycle. Some sulfate is incorporated into sulfated polysaccharides in oxidized form and most into methionine and cysteine. Methionine is converted into DMSP by some marine photoautotrophs (Gage et al., 1997). The synthesis of DMSP is also involved in carbon cycling because each DMSP molecule contains 5 carbon atoms (Sievert et al., 2007). DMSP is the main precursor of DMS, a gas that affects global climate (Salgado et al., 2014). Atmospheric DMS is rapidly oxidized to sulfur-containing acidic aerosols. These aerosols alter the global radiation budget by serving

as “cloud-condensing nuclei” which absorb and scatter the incoming radiation, before falling as acid rain to earth (Yoch, 2002).

1.2 Carbon pump

Oceans are major carbon reservoirs and are known to buffer anthropogenic carbon emissions by drawing CO_2 from the atmosphere and burying it in the deep ocean, a process known as the biological carbon pump (Jiao and Zheng, 2011; De La Rocha and Passow, 2014). This is a process whereby CO_2 in the upper ocean is fixed by photosynthetic primary producers to form organic matter, which is then transported to the deeper ocean by sedimenting particulate organic matter (POM) and the drawdown of dissolved organic matter (DOM) through mixing and ownwelling (Jiao et al., 2010). The carbon pump is of two types i.e. biological carbon pump and physical carbon pump.

1.2.1 Biological process

Phytoplanktons, the primary producers, which are present in the upper layer of the oceans perform photosynthesis which powers carbon fixation in the presence of light. They convert this inorganic carbon to the organic matter. This process, known as primary production, plays a key role in CO_2 withdrawal from the atmosphere and transfer into the marine food web.

Phytoplankton which are responsible for primary production, surprisingly, comprise only a small fraction of the organic carbon pool but, nevertheless, they can generate large amounts of DOM to maintain the entire food chain because of their extremely rapid turnover (Bopp et al., 2007). Apart from phototrophs, heterotrophs also play an important role in this biological process. They remineralize most of this DOM to essential inorganic nutrients which are again used by the primary producers, a process known as the microbial loop. A small portion of this organic carbon, usually the more recalcitrant fraction, migrates from the sea surface to sea floor and is stored in sediments for millions of years (Denman et al., 2007, Ciais et al., 2013). Hence, this biological pump plays an important role in removing this CO_2 from the atmosphere for long periods of time (Figure 1.2.1.1).

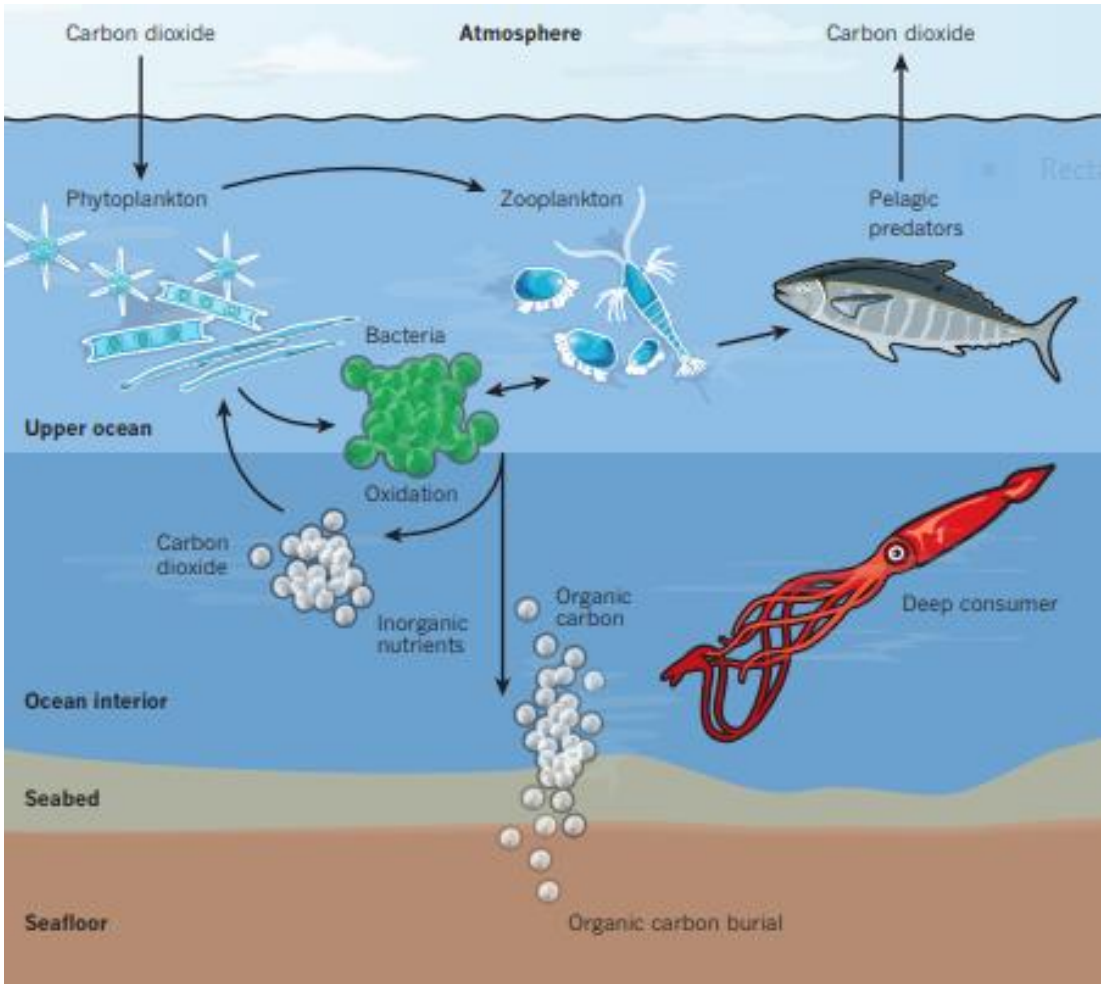

Figure 1.2.1.1. Biological carbon pump showing the role of different micro-organisms in nutrient recycling (Modified from Falkowski, 2012).

1.2.2 Physico-chemical process

Physico-chemical processes are also responsible in increasing the distribution of carbon in the deep sea. Cold seawater increases the solubility of CO_2 and, hence, the mixing of deep cold waters with warmer water from the upper layers of oceans helps to dissolve higher concentrations of inorganic carbon. That is why it is called the solubility pump (Raven and Falkowski, 1999). The physical pump allows the transport of CO_2 to deeper parts of the ocean by downwelling and upwelling (figure 1.2.2.1). During downwelling, the dissolved CO_2 from the upper cold surface of the ocean goes down deep into the ocean where it stays there for longer periods of time. The upwelling brings the cold water from the deep sea back to the upper surface where the water heats up and some of the dissolved CO_2 goes back to the atmosphere (Legendre et al., 2015).

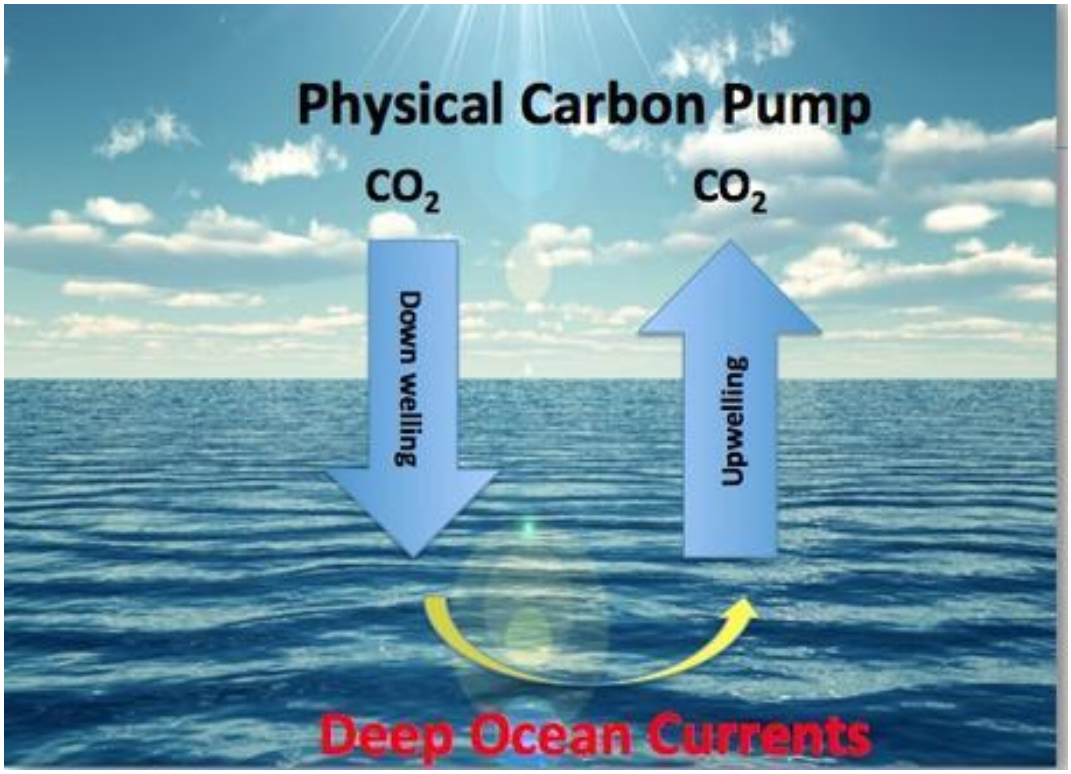

Figure 1.2.2.1: The physical carbon pump showing downwelling and upwelling of the CO_2.

1.3 Types of dissolved organic matter in the ocean

The dissolved organic matter can be classified as:

1.3.1 Labile DOM: The DOM which is taken by heterotrophs in hours or even days after its production.

1.3.2 Semi-labile DOM: Semi-labile DOM stays at the surface of the ocean from weeks to years and this DOM is not very reactive.

1.3.3. Refractory DOM: RDOM is the least reactive of all DOM fractions and is stored in the ocean for decades to centuries (Jiao, Herndl et al., 2010). RDOM acts as a reservoir of carbon and is mainly composed of aliphatic compounds poor in nutrients (i.e. nitrogen, phosphorous and other essential nutrients) and extremely rich in carbon (Hopkinson and Vallino, 2005).

The accumulation of the RDOM occurs due to the transformation of LDOM and SDOM through microbial carbon pump. The carbon to nitrogen and carbon to phosphorus ratios in RDOM is usually very high and ratios changes from the normal/typical C:N:P ratios of 199:20:1 in LDOM to C:N:P = 3,511:202:1 in the recalcitrant fraction (Jiao et al., 2010).

1.4 Hydrolysis of DOM

Around 70% of the DOM in the oceans is considered of low-molecular weight (< 1 kDa) (Benner, 2002). The other 30% of DOM is of high molecular weight (> 1 kDa) and, curiously, is much less refractory and, hence, more readily degraded than the low-molecular weight fraction (Decho and Gutierrez, 2017). Most of the high molecular weight DOM is in the form of biopolymers, and because biological membrane systems are only permeable to molecules smaller than 0.6 kDa (Weiss et al., 1991), exoenzymes or ectoenzymes play a key pivotal role in polymeric DOM hydrolysis and assimilation (Vetter and Deming, 1999) as these biopolymers must be hydrolyzed outside the cell before they can be taken up by most organisms (i.e., non-grazing organisms). The activities of secreted enzymes in marine microbes has generally been assessed through the use of fluorescently labelled substrates (Karner and Herndl, 1992; Martinez et al., 1996; D'Ambrosio et al., 2014; Arnosti, 2015), simple observation of bacterial growth on different polymeric substrates (e.g., Mitulla et al., 2016) or, more rarely, identifying and characterizing the actual enzymes involved in hydrolyzing the DOM (e.g., Hehemann et al., 2014; Xing et al., 2015). Interestingly, marine bacterial exoenzymes are proving highly distinct from their well-characterized terrestrial counterparts (Michel and Czjzek, 2013) and are currently poorly identified. But there are some examples of hydrolysis of the DOM such as *Zobellia galactanivorans* which is a marine flavobacterium used a model organism to study the degradation of algal polysaccharides. It has been reported that *Z. galactanivorans* encodes a large array of CAZymes to degrade algal polysaccharides (Barbeyron et al., 2016). CAZymes actually follow the succession of bacterioplankton communities which follow phytoplankton blooms. Large numbers of CAZymes were detected in Gammaproteobacteria and Flavobacteria during phytoplankton blooms degrading polysaccharides (Teeling et al., 2012, Sperling et al., 2017). In the Gammaproteobacterium, *Alteromonas macleodii* strain 83–1 CAZymes causes the hydrolysis of the DOM and helped this strain to grow on different substrates (Neumann et al., 2015). It has also been detected in the marine strain *Bacillus weihaiensis* Alg07 that it contains

enzymes that can completely degrade the specific polysaccharides of brown algae i.e. laminarin and alginate (Zhu et al., 2016).

1.5. Exoproteomics

Microbial secretion of proteins is a universal phenomenon that allows microbes to modify or otherwise influence their community and environment. By analysing the secreted proteins, we can infer an organism's ecological strategy and its function within the environment (Christie-Oleza, Pina-Villalonga et al. 2012). Exoproteomics is the large - scale study of extracellular proteins of a biological system. Interestingly, up to 35% of bacteria - encoded proteins are predicted to encode proteins secreted from the cell, including membrane-linked proteins like membrane transporters, ectoenzymes, or motility proteins that can easily be lost by the cell and found in the exoproteomic fraction (Armengaud, Christie-Oleza et al. 2012). The term exoproteome is better illustrated in figure 1.5 with a Gram-negative bacterium model. The exoproteome regroups the 'secreted exoproteins' and the 'non-secreted exoproteins'. The secretome regroups the so-called 'secreted exoproteins'. The term 'secretome' refers to the pool of proteins which are actively secreted via classical or nonclassical mechanisms or via the release of exosomes, as well as the secretion machinery itself but, also, of other metabolites (Desvaux, Hebraud et al. 2009). The secreted proteins comprise membrane-linked proteins as well as other translocated proteins. Proteins released into the extracellular milieu belong to the latter group of proteins. A proportion of the exported proteins are inserted in the membrane via the presence of transmembrane hydrophobic segments or can be associated in some cases with other transmembrane-inserted proteins, as exemplified by some components of the flagellum apparatus of motile bacteria (Buttner, 2012). The specific fraction of secreted proteins bound to the external surface of the cell is also known as the 'surface proteome'.

The use of shotgun proteomics has become a powerful tool for detecting the array of proteins present in the extracellular medium of an organism under different experimental conditions (Armengaud et al., 2012) and, hence, a reliable high throughput method for identifying key secreted enzymes and proteins involved in cell-to-cell and cell–environment interactions.

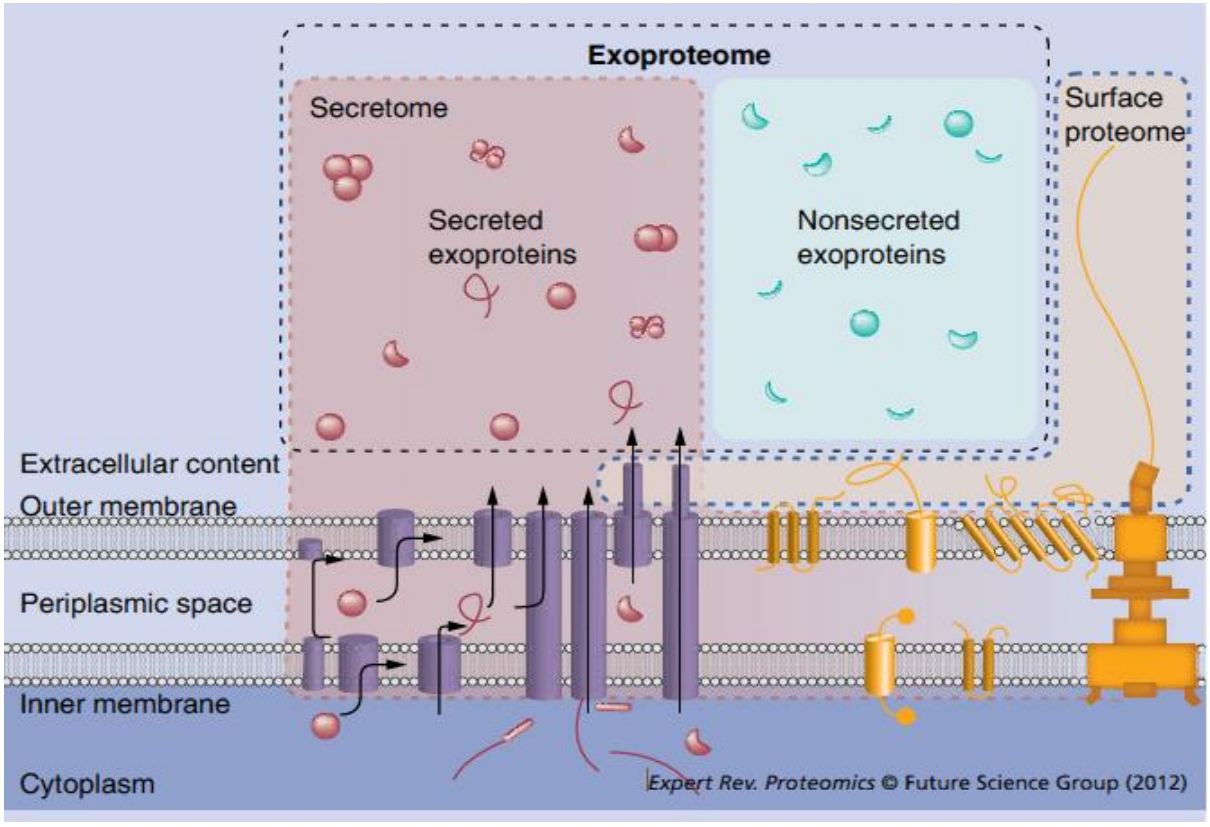

Figure 1.5: Secretome, surfaceome and exoproteome concepts illustrated with a Gram-negative bacterium model (modified from Armengaud, Christie-Oleza et al., 2012).

1.5.1 Secretion systems in Gram-negative bacteria:

Gram-negative bacteria have some protein secretion systems to transport proteins across 1, 2, or 3 phospholipid membranes. These protein secretion systems are T1SSs, T2SSs, T3SSs, T4SSs, T5SSs and T6SSs (figure 1.5.1). Protein secretion systems in several Gram-negative bacteria transport their substrates across both bacterial membranes in a one-step, Sec- or Tat-independent process. These include the T1SSs, T3SSs, T4SSs, and T6SSs. These pathways have periplasm-spanning channels and secrete proteins from the cytoplasm outside the cell, but their protein secretion mechanisms are quite different. The T3SS, T4SS, and T6SS can also transport proteins across an additional host cell membrane and delivers secreted proteins directly to the cytosol of a target cell (Green and Mecsas, 2016). Some proteins are secreted in a two-step, Sec- or Tat-dependent mechanism. These proteins cross the inner membrane with the help of either the Sec or Tat secretion pathways and are then

transported across the outer membrane using a second secretion system. The T2SSs and T5SSs secrete proteins in this manner (Green and Mecsas, 2016).

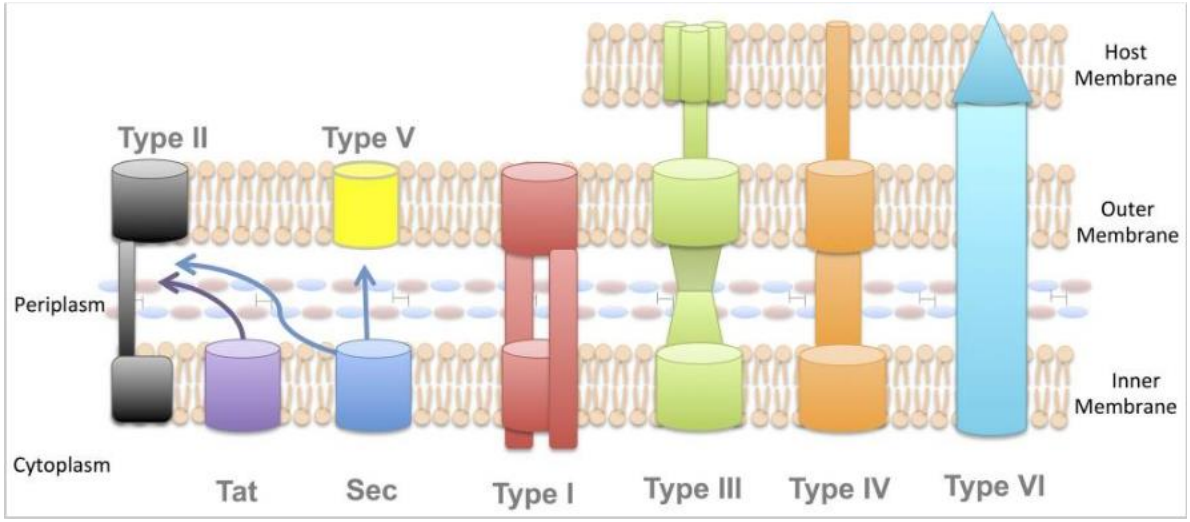

Figure 1.5.1: Secretion systems in Gram-negative bacteria (Modified from Green and Mecsas, 2016).

1.5.2: Secretion systems in Gram-positive bacteria:

A single cytoplasmic membrane is present in Gram-positive bacteria which is surrounded by a very thick cell wall. Gram-positive bacteria by using the Tat and Sec secretion systems secrete proteins across the membrane. Gram-positive bacteria use SecA2 as an additional factor for Sec secretion. They also use “injectosomes” to transport proteins from the bacterial cytoplasm into the cytoplasm of a host cell in a 2-step process (Green and Mecsas, 2016).

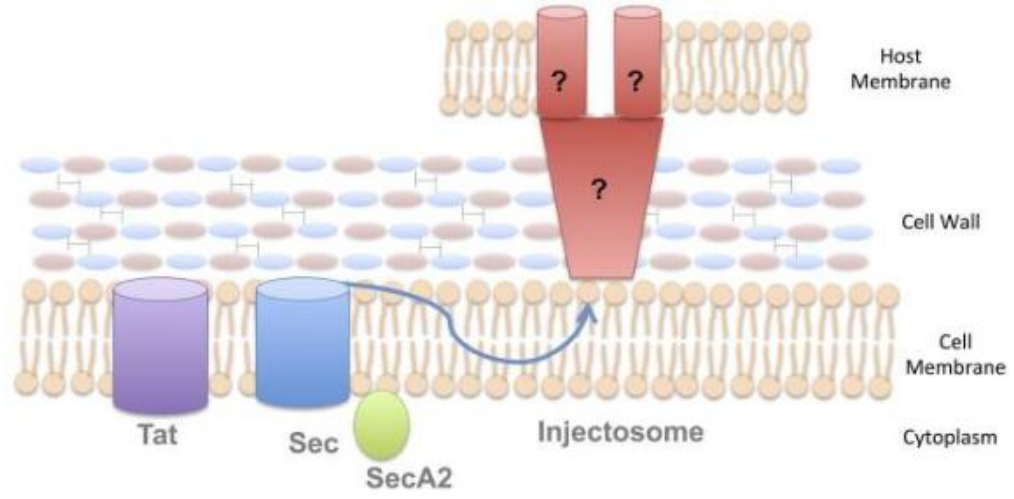

Figure 1.5.2: Secretion systems in Gram-positive bacteria (Modified from Green and Mecsas, 2016).

1.6 Introduction about phytoplankton

Phytoplankton are the photosynthetic micro-organisms that have the capacity to convert carbon dioxide and water into organic mater using energy from light. The different types of phytoplankton that are present in ocean includes cyanobacteria, green algae, diatoms, dinoflagellates and coccolithophores. Diatoms are unicellular algae whose cell wall is made of silica (Hasle et al., 1996). Dinoflagellates consists of two unequal flagella that help in their movement. They contain pigments which also give colour to the water and during their blooms they form red tide or brown tide (Hasle et al., 1996). Coccolithophores are covered with coccoliths which are made of calcium carbonate (Moheimani et al., 2012). Coccolithophores are known for producing dimethyl sulphide gas and are involved in sulfur cycling. Algae are able to make blooms when light and nutrient availability is high. During algal blooms algae concentration can be extremely high and, consequently, produce large ammounts of organic compounds that are required by other microbes and higher organisms (Peter and Huber, 1997, Jacques, 1993).

Phytoplanktons are the main source of carbon and energy in the ocean despite of their low abundance (Jardillier, Zubkov et al., 2010). Cyanobacteria are numerically the most abundant phototrophs in the world's ocean and about a quarter of the global primary production is driven by marine cyanobacteria. Cyanobacteria has two membranes i.e. outer membrane and inner membrane (figure 1.6.1). Cyanobacteria consists of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) which is the key enzyme for photosynthetic CO_2 fixation and is bound within proteinaceous polyhedral microcompartments called carboxysomes (Long et al., 2007). Phycobilisomes act as light-harvesting antennae for the photosystems in cyanobacteria, provides blue-green pigmentation to cyanobacteria and are attached to the thylakoid membrane (Grossman et al., 1993). Marine cyanobacteria, known as picocyanobacteria due to their small size, include two genera, *Prochlorococcus* and *Synechococcus,* both with a common ancestor (Dufresne et al., 2008, Urbach et al., 1998). Due to the orange phycoerythrin fluorescence, *Synechococcus* was detected before of

Prochlorococcus despite the latter being the most dominant genus (Waterbury et al., 1979). Both genera have different pigmentation, their cell size is also different (Moore et al., 2002, 2005) and even the composition of elements is different (Bertilsson et al., 2003). In terms of light harvesting, both genera have different adaptation strategies. *Synechococcus* is able to collect different light qualities because of the presence of a different varities of pigmentation as compared to *Prochlorococcus* which has simple pigmentation and can collect only blue light. Only one pigment is shared by both genera, which is zeaxanthin and it helps in photoprotection (Goericke and Repeta., 1992, Kana et al., 1988). Both contain a single circular chromosome and do not have any plasmid. *Synechococcus* and *Prochlorococcus* have small genome, and its size is 2.2 to ~2.86 Mb in *Synechococcus* and 1.64 to 2.7 Mb in *Prochlorococcus* (Scanlan et al., 2009). Another important feature is that catalase - peroxidase which helps to get rid of oxidative stress is present in all *Synechococcus* strains except BL107, CC9311, CC9902, and WH8102, but is absent in all *Prochlorococcus* strains which are sequenced to date. It has been detected in *Prochlorococcus* strain MIT9215 that it benefits from the catalase produced by surrounding heterotrophs (Morris et al., 2008).

In this project *Synechococcus* sp. WH7803 was used. *Synechococcus* is present in temperate to tropical oceans. This genus was first described in 1979 (Johnson and Sieburth, 1979). Some *Synechococcus* cells have gliding motility (Castenholz, 1982). The main photosynthetic pigment of *Synechococcus* is chlorophyll a, but phycobiliproteins are the major accessory pigments (Waterbury et al., 1979). Phycocyanin, allophycocyanin, allophycocyanin B and phycoerythrin are the 4 commonly recognized phycobilins (Stanier and Cohen-Bazire, 1977). *Synechococcus* cells are very abundant in nutrient-rich environment than in oligotrophic ocean and usually prefers the well-lit upper portion of euphotic zone (Partensky et al., 1999a).

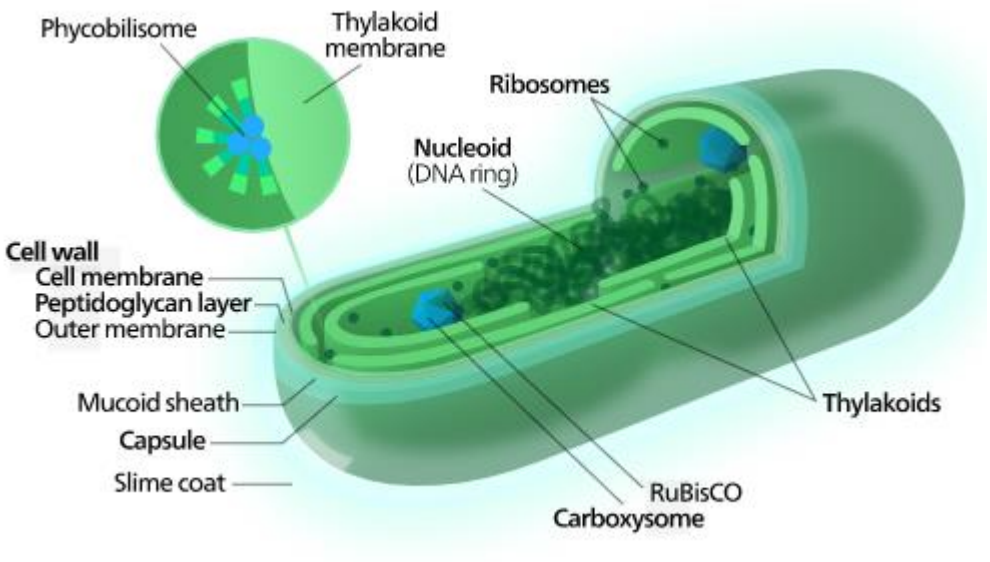

Figure 1.6.1: Cell wall structure of cyanobacteria (modified from wikipedia).

1.7 Introduction of the different heterotrophs used in this PhD thesis

Heterotrophic bacteria play an important role in organic matter cycling in the ocean. The most dominant bacterioplankton in the ocean is the SAR11 group (Morris et al., 2002). They are small, free-living heterotrophic bacteria. They were first discovered in 1990 from the Sargasso Sea (Giovannoni et al., 1990). SAR11 are known for having a proteorhodopsin which is a light dependent proton pump (Giovannoni, 2017). They have some of the most streamlined genome, which helps to reduce the nutrient cost of replication and also helps to increase the transport functions when there are low nutrient concentrations (Giovannoni, 2017). Apart from SAR11 there are other less abundant copiotrophic bacteria in the ocean which are thought to play a much more active role in DOM remineralisation. The different heterotrophs used in this project are shown in table 1.7.1 which contains their name, strain, culture conditions, genome size, GC content and number of CDS. The features of these heterotrophs have been mentioned in table 1.7.2.

1.7.1. Actinobacteria: Actinobacteria are Gram positive bacteria which are aerobic and non-motile. Their genomic GC content is very high (70-80%) (Goodfellow and Williams, 1983). About 10% of the bacteria that colonise marine snow belong to the Actinomycetes group (Alldrege and Silver, 1988). Their presence is maintained by their antagonistic activity which

affects the breakdown and recycling of organic matter (Grossart et al., 2004). This is the reason that they behave as the active component of microbial communities in the marine environment. Various novel secondary metabolites have also been isolated from the marine Actinobacteria (Fedler et al., 2005). More than half of the antibiotics discovered to date are from Actinomycetes (Berdy, 2005). The Actinobacteria that were studied in this project are *Salinispora tropica* CNB-440 and *Aeromicrobium marinum* T2.

1.7.1.1*. Salinispora tropica*: *Salinispora tropica* is a marine bacterium that belongs to Actinomycetes group. It is used as a model to study the adaptations to marine environment as it is was the first Gram- positive bacterium isolated from a marine environment (Mincer et al., 2002). The genus *Salinispora* was described as the first sea-water requiring marine actinomycete (Maldonado et al., 2005). One of the most important characteristic of this bacterium is that it produces unique secondary metabolites which proves that it has an enormous potential for the discovery of new medicines. One of the secondary metabolites detected in *S. tropica* is salinosporamide A which is a proteasome inhibitor and it currently in clinical trials (Feling et al., 2003).

1.7.1.2. *Aeromicrobium marinum*: *Aeromicrobium* is a rod-shaped, obligately salt dependent, Gram positive, non-spore forming, non-motile and aerobic bacterium. This bacterium was first isolated from the German Wadden sea water surface (Bruns et al., 2003). This bacterium contains LL-diaminopimeric acid in its peptidoglycan. *A. marinum* has large amounts of different fatty acids (Bruns et al., 2003). *A. marinum* has been characterised for the large diversity of organic compounds it can grow on e.g. trehalose, cellobiose, crotonic acid, 2-oxoglutarate, succinate, fumarate, mannitol, pyruvate, arginine and glutamic acid, but interestingly, it is unable to degrade starch, cellulose, lipids, chitin and casein (Bruns et al., 2003).

1.7.2. CFB (Cytophaga-Flavobacterium-Bacteroidetes) group: The characteristics of Cytophaga–Flavobacteria-Bacteroidetes is that they are chemoorganotrophic and can degrade various biopolymers such as cellulose, chitin, and pectin (Reichenbach et al., 1991).

Members of Bacteroidetes have been found to be abundant during algal blooms (Pinhassi et al., 2004) and which shows their preference to degrade polymers over monomers (Cottrell and Kirchman, 2000). Previous analysis reveals that Bacteroidetes were found to be enriched on organic matter particles and marine snow (Pedrotti et al., 2009). The Bacteroidetes lifestyle is mainly attachment to surfaces and degradation of polymers as assumed by its free-living and particle associated diversity of bacteria (DeLong et al., 1993). Members of CFB group are marine heterotrophic bacterioplanktons which are commonly present on organic matter and marine snow (De Long et al., 1993, Rath et al., 1998). These organisms are capable of degrading HMW organic matter including both DOM and POM and thus play a major role in carbon cycle in the ocean (Kirchman, 2002). They are found in different types of marine environments i.e. coastal, offshore, sediments and hypothermal vents (Alonso et al., 2007, Pommier et al., 2007). The CFB group can breakdown cell wall, chitin, starch, xylan pectin, agar and laminarin (Mayrberger, 2011), but some members of Bacteroidetes can also break-down cellulose (Thomas, 2011). Cellulose cannot be transported into the cell because of its large size, so it secretes extracellular enzymes that form the complex cellulosome which break-down cellulose and is imported into the cell (Bayer, 1998). This cellulosome consists of exo-glucanase, endo-glucanase, xylanases, and hemicellulose. Hence, Bacteroidetes play a very important role in the breakdown of recalcitrant long-chain DOM in the ocean (Pinhassi et al., 2004, Rieman et al., 2000). These Gram- negative bacteria possess gliding motility and are non-spore forming rods (Kirchman, 2002). This group is characterised by the presence of flexirubin type pigmentation which is produced only by this CFB group (Kirchman, 2002). Members of this group are mostly aerobic except some which are facultative anaerobes (Reichenbach et al., 1991).

The members of this group that were used in this project were: *Polaribacter sp*. MED 152, *Algoriphagus machipongonensis* PR1, *Gramella forsetii* KT0803 and *Formosa agariphila* KMM3901.

1.7.2.1. *Polaribacter sp*.: This aerobic bacterium is from family Flavobacteriaceae. *Polaribacter sp*. MED 152 has been reported to contain some genes which helps in its attachment to various substrates (De Long et al., 1993). These genes also help in their gliding motility. Apart from this, this bacterium is also able to break-down various polymers and can use them as a source of carbon and energy. Despite MED 152 is a model organism to study the role and molecular mechanisms of proteorhodopsins (encoded in its genome), it is unable to grow autotrophically (Gonzalez et al., 2008). Proteorhodopsin is a light dependent proton pump which is involved in ATP synthesis and helps to provide energy to the cells (Stepanauskas and Sieracki, 2007, Kirchman, 2008, Gonzalez et al., 2008, Palovaara et al., 2014).

1.7.2.2 *Algoriphagus machipongonenesis*: *A. machipongonensis* is non-spore forming, non-flagellated and non-motile strain. *A. machipongonensis* does not contain flexirubin pigments, but contains carotenoids + MK-7 as the main menaquinone (Alegado et al., 2013). It was co-isolated from a mud core sample near Hog Island, Virginia, USA along with choanoflagellate *Salpingoe carosetta* ATCC50818.

1.7.2.3. *Gramella forsetii* KT0803: *G. forsetii* is a marine heterotrophic bacterium which is aerobic and belongs to Bacteroidetes. It was first isolated from the surface of the North Sea during a phytoplankton bloom (Eilers et al., 2001). The genome analysis reveals that it contains genes that encodes hydrolytic enzymes and that is why they can easily degrade HMW organic matter and use these polymers as a source of carbon and energy (Bauer et al., 2006). *G. forsetii* KT0803, is the first marine Bacteroidetes whose whole genome analysis showed that it contains substantial number of CAZymes and peptidases (Bauer et al., 2006). The proteomic analysis proved the presence of PUL systems i.e. starch, laminarin and alginate PUL (Kabisch et al., 2014). *G. forsetii* is present in coastal regions in the ocean (Bauer et al., 2006).

1.7.2.4. *Formosa agariphila* KMM3901: *F. agariphila* belongs to Bacteroidetes and is a facultative anaerobe. It was first isolated from the green alga *Acrosiphonia sonderi* from

Troitsa Bay in the Gulf of Peter the Great from the Sea of Japan (Nedashkovskaya et al., 2006). It is known to be a specialist in the breakdown of proteins, polysaccharides and glycoproteins because its genome contains 129 proteases and 88 glycoside hydrolases. *F. agariphila* has an alga-associated lifestyle because they degrade different varities of algal polysaccharides from green, red and brown algae (Mann et al., 2013).

1.7.3. <u>Alphaproteobacteria:</u> Marine Alphaproteobacteria are mainly composed of the SAR11 group (numerically dominant group amongst the marine heterotrophic community, but extremely specialised and hard to grown in laboratory conditions) and the more generalist lifestyle Roseobacter lineage. Apart from the numerically dominating marine Alphaproteobactium SAR11, Roseobacters form a well-supported clade in the Alphaproteobacteria. The Roseobacter clade is very abundant in the ocean and can constitute upto 20% of bacterial cells in some coastal regions and 3-5% of bacterial cells in surface water of open ocean (Moran et al., 2007). The members of Roseobacter clade are ubiquitous in temperate and polar oceans (Selje et al., 2004). Roseobacters plays an important role in the carbon and sulphur cycling (Geng and Belas, 2010). The strains from the Roseobacter clade that were used in studying the phototroph-heterotroph interactions are mentioned below.

1.7.3.1. *Ruegeria pomeroyi* DSS-3: *R. pomeroyi* was first isolated from the coastal Georgia seawater in 1998 and was capable of breaking DMSP (Gonzalez et al., 2003) and was the first Roseobacter to have its genome sequenced (Moran et al., 2004). *R. pomeroyi* is a moderate copiotroph. It is also used as a model organism to study the biogeochemical, ecological and physiological strategies of other marine heterotrophic bacteria (Moran et al., 2004, Burgmann et al., 2007, Lauro et al., 2009). Many ABC and TRAP transporters were also detected in the genome of *R. pomeroyi* (Christie-Oleza and Jean Armenguad, 2010). Among the Roseobacter lineage, *Ruegeria pomeroyi* has become a model organism for studying key molecular processes in marine ecosystems due to its versatility and it being the first member of the clade with its genome sequenced (Christie-Oleza and Armengaud, 2015).

1.7.3.2. *Roseobacter denitrificans* OCh114: *R. denitrificans* was first isolated in Australia from coastal marine sediments. *Roseobacter denitrificans* is another widely used model organism as it generates energy by using aerobic anoxygenic photosynthesis to generate an extra supply of energy (Wagner-Dobler and Biebl, 2006). It is used as a model organism to study the importance of aerobic anoxygenic photosynthesis (AAnPs) in the ocean (Swingley et al., 2007). AAnPs means photosynthesis without the production of oxygen, but in the presence of oxygen. About 15% of *R. denitrificans* genome consists of transport proteins (Swingley et al., 2007).

1.7.3.3. *Dinoroseobacter shibae* DFL-12: *D. shibae* was first isolated from the toxic marine dinoflagellates cultures from the Bay of Tokyo (Shiba et al., 1979). *D. shibae* consists of a carotenoid with spheroidenone due to which it is dark red in colour. It is also able to perform AAnP (Shiba, 1991). It is motile because of the presence of single, polarly inserted flagellum. It also produces AHL which is a quorum sensing signalling compound. It can also produce polymeric capsule in culture (Patzelt et al., 2013). It contains 7 plasmids which have 861Kb of extrachromosomal information. *D. shibae* is capable of synthesizing vitamin B1 and vitamin B12 and, hence, can supplement auxotrophic algae. Two pathways for the de novo synthesis of vitamin B12 have been described. One of which is oxygen dependent and other is oxygen independent pathway. It provides vitamin B1 and vitamin B12 to its dinoflagellate host *Prorocentrum minimum* (Wagner Dobler et al., 2010) and they both depend on each other for exchange of compounds (Wang et al., 2014).

1.7.4. Gammaproteobacteria: The heterotrophs that belonged to Gammaproteobacteria and were used in this project are described below.

1.7.4.1. *Pseudoalteromonas citrea* NCMB1889: *P. citrea* is yellow-pigmented and can produce antibiotics. It was first isolated from the Mediterranean water off Nice (Gauthier, 1977). *P. citrea* is the reclassified name of the former *Alteromonas citrea* (Gauthier et al., 1995). *Pseudoalteromonas* species are found associated with some eukaryotic hosts in the marine environment and play very important role in the understanding the microbe-host

interactions. *P. citrea* has anti-bacterial, anti-fungal and agarolytic activities (Holmstrom and Kjelleberg, 1999).

1.7.4.2. *Alteromonas macleodii* ATCC27126: *A. macleodii* was first isolated from waters off Oahu, HI (Baumann et al., 1972). This is one of the first isolates that was described a marine bacterium. It is strictly aerobic. *A. macleodii* has motility due to the presence of one polar flagellum. It can grow on glucose as a source of carbon and energy. It also needs large amount of sodium for its growth like other marine microbes (Gonzaga et al., 2012). It is globally distributed from temperate to tropical marine environments (Garcia-Martinez et al., 2002). In the ocean where there is a high concentration of nutrients it becomes very abundant (Schafer et al., 2000) and even in marine snow and on POM.

1.7.4.3. *Marinobacter adhaerens* HP15: *M. adhaerens* was first isolated from the German Wadden Sea as a particle associated marine bacterium (Grossart et al., 2004). *M. adhaerens* has the ability to grow on different media heterotrophically, and genetically it can be easily accessed. It is also used as a model organism to study the interaction with the diatom *Thalassiosira weissflogii* (Gardes et al., 2010) to which it can attach forming marine snow particles by inducing exopolymer and aggregate formation (Gardes et al., 2010). It also encodes flagella associated genes (Gardes et al., 2010).

1.7.4.4*. Pseudomonas stutzeri* AN10: *P. stutzeri* presents a single polar flagellum. *P. stutzeri* was first isolated from polluted marine sediments and is capable of degrading naphthalene (Garcia-Valdes et al., 1988). It consists of catabolic genes which can degrade naphthalene (Bosch et al., 1999a) and these catabolic genes are organised in upper pathway operon, lower pathway operon and the regulatory gene. Apart from this, *P. stutzeri* also contains additional salicylate hydroxylase gene in its genome.

1.7.5. Planctomycetes: Planctomycetes clade is widely distributed in marine environment and is involved in global carbon and nitrogen cycles. They are capable of anaerobic oxidation of ammonium by using anammoxosomes (Lindsay et al., 2001, Van Niftrik et al., 2004), which

plays an important role in the loss of fixed nitrogen in marine oxygen minimum zones (OMZs). The two genera that were studied in this project were *Planctomyces limnophilus* ATCC43296 and *Rhodopirellula baltica* SH1.

1.7.5.1. *Planctomyces limnophilus*: *P. limnophilus* was first described by Hirsch and Muller in 1986. It belongs to family Planctomycetaceae. This bacterium is aerobic, Gram negative, spherical to ovoid, non-sporulating with a sub-polar flagellum (Hirsch and Muller, 1986). It is very different from other bacterial taxa due to some reasons, i.e. by its cell compartmentalization internally and a cell wall which do not have a peptidoglycan layer but instead it consists of layer which is proteinaceous (LaButti et al., 2010).

1.7.5.2. *Rhodopirellula baltica*: *R. baltica* is a marine bacterium which is distributed globally. It is an aerobic heterotroph that belongs to the environmentally important bacterial order Planctomycetales. *R. baltica* has the largest known circular genome. It was first isolated from the water column in the Kiel Fjord (Baltic Sea) (Schlesner et al., 2004). Its genome analysis reveals that it contains sulfatase genes, carbohydrate-active enzymes (Cantarel et al., 2009) and a pathway for C1 metabolism (Glockner et al., 2003). *R. baltica* is capable of sessile lifestyle, which is very helpful in biotechnological processes during the production of secreted products (Wecker et al., 2010).

1.7.6. Verrucomicrobia: Verrucomicrobia is abundant within the environment and widely distributed. Verrucomicrobia plays very important role in understanding the evolution of bacteria. On the basis of 16S rRNA gene library studies 6 monophyletic subdivisions have been recognized within phylum Verrucomicrobia (Hugenholtz et al., 1998, Vandekerckhove et al., 2000). Some members of Verrucomicrobia have been recently found that can do the oxidation of methane and then use methane as a source of carbon and energy. This makes them as aerobic and extreme acidophilic methanotrophs (Dunfield et al., 2007, Pol et al., 2007) and helps to understand the methanotrophy evolution and biochemistry of C1 transfer.

Table 1.7.1: Table displaying the information about the group and name of heterotrophs, their strains, culture, isolation, genome size in Mb, GC content (%), and number of coding domain sequences.

Group	Name of organism	Strain	Culture	Isolation	Genome size (Mb)	GC content (%)	No. of CDS
Actinobacteria	*Salinispora tropica*	CNB-440	DSM44818	coarse beach sand/ ocean sediments	5.18	69.5	4522
	Aeromicrobium marinum	T2	DSM15272	surface waters	3.08	71.1	3022
CFB group	*Polaribacter sp.*	MED152	Pinhassi	0.5 m depth, 1 km off the Blanes Bay	2.96	30.6	2615
	Algoriphagus machipongonensis	PR1	ATCC BAA-2233/DSM24695	mud core with a colonial choanoflagellate	4.79	38.7	3968
	Gramella forsetii	KT0803	Marga Bauer	phytoplankton bloom	3.8	36.6	3368
	Formosa agariphila	KMM3901	DSM15362	isolated from alga Acrosiphonia sonderi	4.23	33.6	3482
Alphaproteobacteria	*Ruegeria pomeroyi*	DSS-3	DSM15171	Coastal sea water Atlantic (salinity = 31; 1999)	4.6	64.05	4306
	Roseobacter denitrificans	OCh114	ATCC 33942	green seaweeds in coastal marine sediments	4.32	58.9	3986
	Dinoreseobacter shibae	DFL-12	DSM 16493	Prorocentrum lima (benthic dinoflagellate)	4.42	65.5	4147
Gamma proteobacteria	*Pseudoalteromonas citrea*	NCMB1889	DSM8771	seawater	5.32	41.1	4458
	Alteromonas macleodii	ATCC27126	DSM6062	surface waters (1972)	4.65	44.7	3799
	Marinobacter adhaerens	HP15	DSM23420	surface waters aggregates	4.65	56.82	4006
	Pseudomonas stutzeri	AN10	CCUG 29243	polluted marine sediments	4.29	63.3	4186
Planctomycetes	*Planctomyces limnophilus*	ATCC43296	DSM3776	Surface water	5.5	53.7	4294
	Rhodopirellula baltica	SH1	DSM10527	Brackish water	7.15	55.4	7325
Verrucomicrobia	*Verrucomicrobiae bacterium*	DG1235	David Green	isolated from a dinoflagellate/deep sea/soil	5.78	54.3	4519

Table 1.7.2: Table displaying the features of different heterotrophs i.e. Gram test, motility, shape, growth and +/- of proteorhodopsin.

List of heterotrophs	Gram test	Motility	Shape	Growth	Proteorhodopsin (+/-)
Salinispora tropica	+	Non-motile	rod	Aerobic	-
Aeromicrobium marinum	+	Non-motile	rod	Aerobic	-
Polaribacter sp.	-	Non-motile	rod	Aerobic	+
Algoriphagus machipongonensis	-	Non-motile	rod	Aerobic	-
Gramella forsetii	-	Motile	rod	Aerobic	-
Formosa agariphila	-	Motile	rod	Facultative anaerobe	-
Ruegeria pomeroyi	-	Motile	ovoid to rod	Aerobic	-
Roseobacter denitrificans	-	Motile	ovoid to rod	AAnP	-
Dinoreseobacter shibae	-	Motile	ovoid	Facultative anaerobe	-
Pseudoalteromonas citrea	-	Motile	rod	Aerobic	-
Alteromonas macleodii	-	Motile	curved rod	Aerobic	-
Marinobacter adhaerens	-	Motile	rod	Aerobic	-
Pseudomonas stutzeri	-	Motile	rod	Aerobic	-
Planctomyces limnophilus	-	Motile	spherical to ovoid	Aerobic	-
Rhodopirellula baltica	-	Non-motile	ovoid	Aerobic	-
Verrucomicrobiae bacterium	-	Non-motile	irregular	Aerobic	-

1.8 Marine microbial interactions

Primary produced organic matter by phytoplankton in the ocean is divided into two types: POM and DOM. Phytoplanktons releases large amount of organic matter either by leakage, inefficient grazing or viral lysis (Biller et al., 2014; Christie-Oleza et al., 2015a; Grossowicz et al., 2017), which they are not particularly good at remineralising by themselves. In a closed system, axenic cultures of *Synechococcus* die due to the accumulation of organic matter which becomes toxic (Chirstie-Oleza et al., 2017). Nevertheless, in a co-culture with heterotrophs theses culture survive because the heterotrophs remineralize most of this primary produced organic matter, recycles essential elements like nitrogen, phosphorus, and trace-metals (Christie-Oleza et al., 2017), a key evolution to niche partitioning within the nutrient-poor ecosystems (Azam, 1998). So, interactions between the phototrophs and the heterotrophs are essential in maintaining the nutrient balance. Other theories such as the Black Queen Hypothesis (Morris et al., 2012) base microbial interactions on the development of auxotrophis complemented by the co-occuring organisms although we believe these are simply secondary traits that evolve from the main interactions that are based on nutrient partitioning and exchange between the well-defined groups of organisnms, i.e. phototrophic and heterotrphic organisms.

The Roseobacter group generally shows a positive correlation with the phytoplankton community and forms intimate associations with specific phytoplankton groups (Giebel et al., 2011; Morris et al., 2012). Interestingly, marine Roseobacter strains present a large genomic capability and metabolic versatility to use an array of organic substrates found within phytoplankton exudates and, hence, they are one of the first bacterioplankton groups to react to the input of organic matter produced, for example, during phytoplankton blooms (Newton et al., 2010; Romera-Castillo et al., 2011; Christie-Oleza et al., 2012a; Buchan et al., 2014; Landa et al., 2016; Simon et al., 2017). The strain *Ruegeria pomeroyi* DSS-3 was the first roseobacterium to have its genome sequenced (Moran et al., 2004) and has served as a model organism to study biogeochemical, ecological and physiological strategies of this group

of heterotrophic marine bacteria (Christie-Oleza and Armengaud, 2015) and, for this reason, it is the strain used during this thesis as a model heterotroph.

Marine microbial interactions are also based on vitamin exchange and alleviation of oxidative stress according to Black Queen hypothesis (Morris et al., 2012) and on iron. *Prochlorococcus* lacks catalase and it has to depend on the helper strain, i.e. heterotrophs, to decompose hydrogen peroxide (Morris et al., 2008). The interaction between *Ruegeria pomeroyi* DSS-3 and diatom *Thalassiosira pseudonana* CCMP1335 is based on vitamin exchange. *R. pomeroyi* provides the essential vitamin B12 to its diatom host which in turns provide fixed carbon to *R. pomeroyi* (Durham et al., 2014). *Marinobacter* and the dinoflagellate *Scrippsiella trochoidea* has the beneficial interaction which is based on the exchange of Fe2+, (Amin et al., 2009).

1.9 Hypothesis and aims

The hypothesis of this project is that marine microbial interactions are based on nutrient recycling and exoenzymes play an important role within this process by degrading the DOM produced by phototrophs. To prove this hypothesis *R. pomeroyi* and *Synechococcus* were used as model organisms and co-cultured for 100 – days to identify exoenzymes that are involved in interactions. Different heterotrophs were also tested with *Synechococcus* to identify an array of secreted proteins in different phylogenetic groups.

The overall aim of this thesis was to improve our understanding for marine phototroph – heterotroph interactions. The aims of the different chapters have been discussed below:

1. The main aim of chapter 3 was to identify hydrolytic enzymes and interaction proteins that degrade the DOM produced by *Synechococcus*.
2. The aim of chapter 4 was to investigate how marine microbes from different phylogenetic groups interact with the DOM produced by marine phototrophs and to study the relationship between photoautotrophic and heteotrophic bacteria.
3. The overall aim of chapter 5 was to characterise the hydrolytic enzymes and interaction proteins and to determine their potential role in marine nutrient recycling. This chapter also aimed to study the substrates for some of these hydrolytic enzymes.

Chapter 2

Materials and methods

2.1 Bacterial strains, their growth and experimental setup

2.1.1. Synechococcus WH7803 and R. pomeroyi DSS-3 co-culture experimental set up for Chapter 3.

Marine *Synechococcus* sp. WH7803 was grown in ASW (Wilson et al., 1996) at 22°C at a light intensity of 10 µmol photons m^{-2} s^{-1} with shaking (140 r.p.m.). Roseobacter strain *R. pomeroyi* DSS-3 was grown in marine broth (Difco, France) at 28°C until stationary phase. *Ruegeria pomeroyi* and *Synechococcus* cells were harvested via centrifugation and washed twice in filter-sterilized autoclaved seawater (SW, natural seawater collected from the Gulf Stream in the Gulf of Mexico; provided by Sigma, USA) prior to co-inoculating both organisms in 100 ml ASW (table 7.1) and SW at cell concentrations of ~10^8 and 10^7 cell ml^{-1}, respectively. ASW and SW co-cultures were incubated for up to 100 days in optimal conditions for *Synechococcus*. The triplicate flasks were allowed for each one of the eight time points analyzed, i.e., days 1, 3, 7, 14, 21, 32, 60 and 100. At each time point, *Synechococcus* cell abundance was monitored by flow cytometry (BD FACScan), while viable heterotrophs were counted by colony forming units on marine agar (Difco, France) as previously reported (Christie-Oleza et al., 2017).

For motility visualization, *R. pomeroyi* was grown in 10 ml of marine broth for 40 h after which cells were washed in filter-sterilized autoclaved SW by centrifugation, re-suspended in 10 ml of SW and further incubated for 4 days to starve the cells. Then, 100 ml of starved cells were added to 100 ml of ASW supplemented with 2.5 mM of $(NH_4)_2SO_4$ and 4 × the standard concentration of f2-media vitamin mix. Succinate, glucose, yeast extract (Merck, Germany) and Bacto peptone (Merck, Germany) were added at final concentrations, 0.005%, 0.01%, 0.02%, 0.05% and 0.1% (wt/vol), and incubated for 4 h before imaging cell motility using concave microscope slides under a 100 × objective of a light microscope (Nikon Eclipse Ti) equipped with a widefield camera (Andor Zyla sCMOS). Quantification of motility was based on the number of moving cells observed in 10-s videos obtained from three different fields per condition.

2.1.2. Experimental set up of 16 different heterotrophs grown in the presence/absence of Synechococcus WH7803.

The bacterial strains used were *Synechococcus* sp. WH7803 as a photoautotroph and 16 different heterotrophs (table 2.1.2.1). *Synechococcus* sp. WH7803 was grown in ASW (as mentioned in section 2.1.1) and the 16 different heterotrophs were grown in the media and the temperature as shown in table 2.1.2.1 with shaking at 180 rpm until early stationary phase. Two conditions were used: mono-culture of each of the heterotrophs; and co-culture of the heterotroph with the phototroph *Synechococcus* sp. WH7803. The co-cultures were set up in ASW media and optimal growth conditions for *Synechococcus* sp. WH7803. The heterotroph mono-cultures were grown in identical conditions although the ASW media was supplemented with 0.1% w/v pyruvate as a source of carbon as well as 0.005% w/v yeast extract as a source of vitamins and growth factors, and ammonia (5 mM of $(NH_4)_2SO_4$) since ASW contains only NO_3^- which is not always assimilated by all heterotrophs. Each condition was set up in triplicate and allowed to grow for 3 days at 23°C shaking at 140 rpm. At the start of theexperiment (T0) and on day 3 (T3), *Synechococcus* cell abundance was monitored by flow cytometry (BD FACScan), while viable heterotrophs were counted by colony forming units on marine agar (Difco, France) as previously described (Christie-Oleza *et al*., 2017).

Table 2.1.2.1: List of 16 different heterotrophs showing their names, strains, media and the temperature required for their growth.

Organisms	Strains	Medium	Temp°C
Salinispora tropica	CNB-440	Marine Broth	28
Aeromicrobium marinum	T2	Marine Broth	28
Polaribacter sp	MED152	Marine Broth	28
Algoriphagus machipongonensis	PR1	Marine Broth	28
Gramella forsetii	KT0803	Marine Broth	28
Formosa agariphila	KMM3901	Marine Broth	21
Ruegeria pomeroyi	DSS-3	Marine Broth	28
Roseobacter denitrificans	OCh114	Marine Broth	28
Dinoroseobacter shibae	DFL-12	Marine Broth	28
Pseudoalteromonas citrea	NCMB1889	Marine Broth	26
Alteromonas macleodii	ATCC27126	Marine Broth	26
Marinobacter adhaerens	HP15	Marine Broth	37
Pseudomonas stutzeri	AN10	LB Broth	30
Planctomyces limnophilus	ATCC43296	PYGV agar	30
Rhodopirellula baltica	SH1	Pirellula medium	25
Verrucomicrobiae bacterium	DG1235	Marine Broth	28

2.2 Preparation of exoproteome samples

The exoproteomes contained in the culture milieu were collected after removing all cells via centrifugation at 3220 × g for 15 min at room temperature and further filtering the supernatant through 0.22 µm pore size filters (Millex-GV; Millipore, Germany). A total of 40 and 80 ml of the supernatant of SW and ASW cultures from time course experiment, and 40 ml of supernatant from 16 heterotroph cultures, were used for trichloro-acetic acid precipitation as described previously (Christie-Oleza and Armengaud, 2010). The resulting protein pellets were dissolved in LDS loading buffer (Invitrogen, USA), and the equivalent of 20 ml of ASW cultures and 40 ml of SW cultures (Chapter 3) and cultures from mono-culture and of co-culture of 16 heterotrophs with *Synechococcus* (Chapter 4) were loaded on a precast Tris-Bis NuPAGE gel (Invitrogen, USA) using 1 × MOPS solution (Invitrogen, USA) as the running buffer. SDS-PAGE was performed for a short gel migration (5 mm).

2.3 Trypsin in-gel proteolysis and nanoLC-MS/MS analysis

Polyacrylamide gel bands containing the exoproteome were excised and standard in-gel reduction with dithiothreitol and alkylation with iodoacetamide were performed prior to trypsin (Roche, Switzerland) proteolysis (Christie-Oleza and Armengaud, 2010). The resulting tryptic peptide mixture was extracted using 5% formic acid in 25% acetonitrile and concentrated at

40°C in a speed-vac. For mass spectrometry, the samples were resuspended in 2.5% acetonitrile containing 0.05% trifluoroacetic acid and filtered using a 0.22 µm cellulose acetate spin column 16,000 g for 5 min in order to eliminate undissolved aggregates. Sample were analyzed by means of nanoLC-ESI-MS/MS using an Ultimate 3000 LC system (Dionex-LC Packings) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, USA) using a 60 min LC separation on a 25 cm column and settings as previously described (Christie-Oleza et al., 2015b).

2.4 Data analysis

Raw files were processed using the software package for shotgun proteomics MaxQuant version 1.5.5.1 (Cox and Mann, 2008) to identify and quantify protein using the UniProt databases of *Synechococcus* sp. WH7803 and of 16 different heterotrophs. Samples were matched between runs. Other parameters were set by default. The bioinformatic analysis pipeline was completed using the software Perseus version 1.5.5.3. Decoy and contaminants were removed. The relative protein abundance was obtained from the raw protein intensities from each sample after normalization to protein size and prior to converting to a logarithmic scale with base 2 (Murugaiyan et al., 2016). The missing values were imputed using default parameters. The protein quantification and calculation of statistical significance were carried out using two-sample Student's t test (P = 0.05) using a permutation-based false discovery rate (q = 0.05). Protein categorization was based on KEGG annotations with manual curation using the Conserved Domain search tool from NCBI. Prediction of secreted proteins was carried out using the servers SignalP 4.1 (Petersen et al., 2011), SecretomeP 2.0 (Bendtsen et al., 2005), LipoP 1.1 (Juncker et al., 2003) and PSORTb (Yu et al., 2010). Only those proteins were selected and analysed in thesis that have signal peptides, secreted by non-classical secretion method and have abundance above 0.1. The proteins were first sorted on the basis of signal peptides, rest of proteins were sorted on the basis of non-classical secretion and left-over proteins were sorted on the basis of abundance above 0.1. This selection criteria was applied to both chapter 3 and chapter 4 for the selection of proteins. This selection criteria

was not applied to catalase/peroxidase proteins and all of these proteins were taken into account in all the analysis.

2.5 Knockout mutants

2.5.1. Detection of secreted proteins and the methods to get mutants

The time course experiment (Chapter 3) highlighted 13 proteins with a potential role in the phototroph-heterotroph interaction (table 2.5.1.1). Plasmid vectors to generate the knock-out mutations by double-homologous recombination were generated via two different methods: i) endonuclease restriction and ligation method for genes 2 and 3; and ii) Gibson assembly method (Gibson et al., 2009) for genes 1, and 4 to 9. Mutants 10 to 13 were provided by others as indicated in Table 5.2.1.1.

Table 2.5.1.1: List of secreted proteins detected from time course experiment showing their accession numbers, their function, categories and the names given to them.

List of secreted proteins		Description	General category	Names
AAV93776.1	YP_165722.1	hypothetical protein SPO0459 (Pectate lyase)	Hydrolase	Protein 1
AAV93680.1	YP_165625.1	type I secretion target repeat protein	Interaction	Protein 2
AAV95476.1	YP_167436.1	BNR/Asp-box repeat domain protein (Sialidase)	Hydrolase	Protein 3
AAV93800.1	YP_165745.1	microcystin dependent protein, putative	Interaction	Protein 4
AAV95658.1	YP_167620.1	type I secretion target repeat protein	Interaction	Protein 5
AAV96145.1	YP_168112.1	Ser/Thr protein phosphatase/nucleotidase, putative	Hydrolase	Protein 6
AAV96272.1	YP_168240.1	metallo-beta-lactamase family protein	Hydrolase	Protein 7
AAV97448.1	YP_165143.1	amidohydrolase domain protein (plasmid)	Hydrolase	Protein 8
AAV95890.1	YP_167855.1	protease, S2 family	Hydrolase	Protein 9
AAV95139.1	YP_167097.1	twin-arginine translocation pathway signal sequence domain protein (PhoX)	Hydrolase	Protein10
AAV96685.1	YP_168655.1	flagellin protein	Interaction	Protein 11
AAV95529.1	YP_167489.1	portal protein, HK97 family (GTA)	Interaction	Protein 12
AAV93552.1	YP_165496.1	PaxA, putative	Interaction	Protein 13

2.5.1.1 Mutations based on restriction sites

Fragments of the genes labelled as AAV93680.1 and AAV95476.1 proteins in table 2.5.1.1 were amplified by PCR from genomic DNA extracted from *R. pomeroyi* and cloned in the plasmid pGP704 (figure 2.5.1.1.1). The plasmid pGP704 is a suicidal plasmid derivative of plasmid pBR322 (Miller and Mekalanos, 1988). pGP704 conserves the ampicillin resistance but has a deletion of the pBR322 origin of replication (*oriE1*) that carries, instead, a cloned fragment containing the origin of replication of plasmid R6K. The R6K origin of replication (*oriR6K*) requires for its function a protein called pi, encoded by the *pir* gene and, hence, can only replicate in the *E.coli* S17-1 λpir strain. In this strain the pi protein is supplied in trans by a prophage (lambda pir) that carries a cloned copy of the pir gene. This strain has chromosomally integrated conjugal transfer functions (RP4 transfer functions). The plasmid also contains a 1.9-kb *BamHI* fragment encoding the *mob* region of RP4. Thus, pGP704 is mobilized into the recipient strain by type RP4 conjugation. Next, the antibiotic cassette gentamicin (Gm) from the plasmid p34S-Gm (figure 2.5.1.1.2; (Dennis and Zylstra, 1998)) was cloned into the gene fragments as shown in figure 2.5.1.1.1 and detailed below.

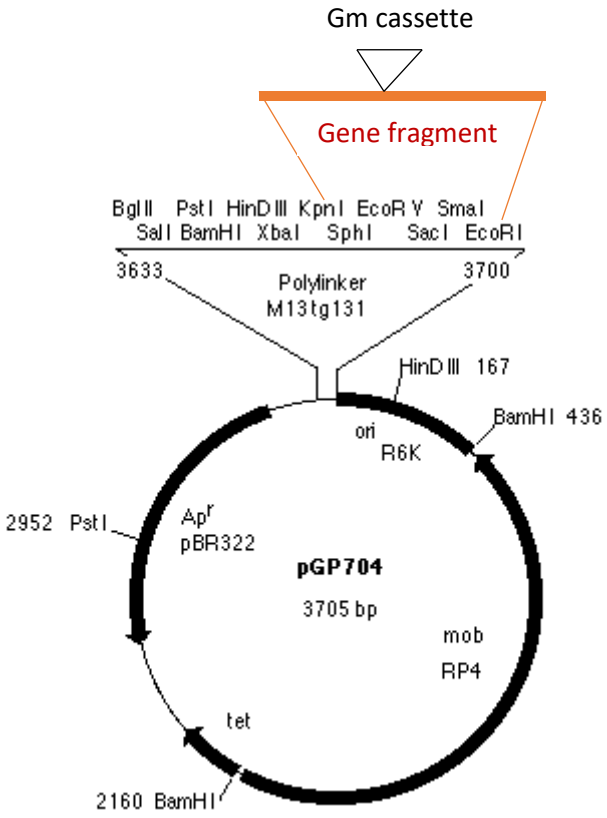

Figure 2.5.1.1.1: Plasmid pGP704 with the gene fragment (2 gene fragments) and the Gm cassette from the plasmid p34S (Dennis and Zylstra, 1998).

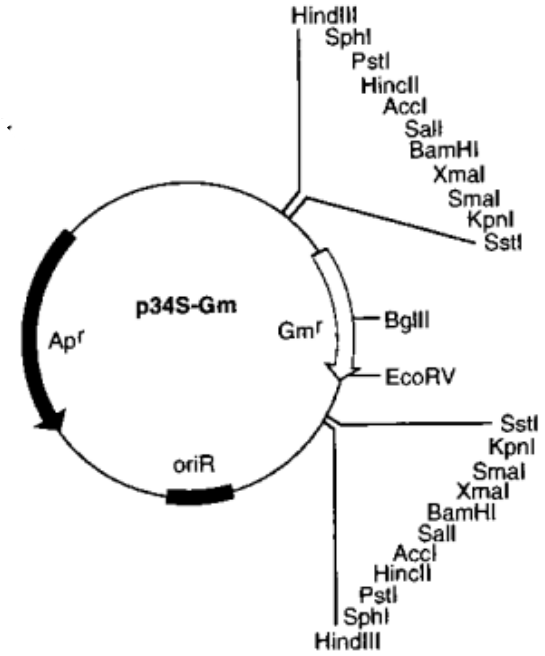

Figure 2.5.1.1.2: plasmid p34S-Gm (Dennis and Zylstra, 1998).

From miniprep 40 µl of a plasmid p34S-Gm was combined with 5 µl of FastDigest buffer and 5 µl of FastDigest restriction enzyme SalI to form 50 µl reaction mixture. Reaction mixture was incubated at 37°C for 30 minutes. Digested reaction mixture was purified according to QIA quick PCR purification Kit (QIAGEN) protocol. The purified reaction mixture of 40 µl was combined with 5 µl of FastDigest buffer and 5 µl of FastDigest SmaI restriction enzyme to form a 50 µl reaction mixture. Reaction mixture was incubated at 37°C for 30 minutes. The plasmid p34S-Gm was first digested with SalI and then with SmaI to avoid auto-ligation because SalI has sticky ends and SmaI has blunt ends.

The 50 µl of the plasmid pGP704 containing the gene fragments for proteins 2 and 3 was combined with 5 µl of FastDigest buffer and 5 µl of FastDigest restriction enzymes to form 50 µl reaction mixture. The reaction mixture was incubated at 37°C for 30 minutes. The restriction enzymes for these 2 proteins were EcoRV.

Digested Gm cassette and pGP704 derivatives were purified prior to ligation according to the Pure Link™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) protocol.

Blunt-end SmaI-digested Gm cassette and blunt-end EcoRV-digested pGP704 derivative plasmids were ligated. The 7 µl of the digested and purified Gm cassette and 7 µl of digested and purified pGP704 derivative plasmids were combined with 4 µl of T4 DNA ligase buffer and 2 µl of T4DNA ligase to form a 20 µl reaction mixture. Reaction mixture were held at 14°C overnight in a PCR machine.

The ligation product was transformed in *E. coli* S17-1λpir competent cells. LB agar plates amended with ampicillin and gentamicin were used to select the colonies that had incorporated the plasmid constructs.

Plasmids were extracted using the miniprep protocol from cell culture originating from transformants. The size of the plasmid construct was compared to that of the pGP704 original plasmid by agarose gel electrophoresis. The constructs were checked by Sanger sequencing.

2.5.1.2 Mutations using Gibson assembly

The genes from proteins 1 and 4 to 9 were knocked out using the Gibson assembly method which is described below (Gibson et al., 2009). Gibson Assembly efficiently joins multiple overlapping DNA fragments in a single-tube isothermal reaction. The Gibson Assembly Master Mix includes three different enzymatic activities that perform in a single buffer. These three enzymes are exonuclease, DNA polymerase and DNA ligase. The exonuclease creates single-stranded 3´ overhangs that facilitate the annealing of fragments that share complementarity at one end (overlap region). The DNA polymerase fills in gaps within each annealed fragment. The DNA ligase seals nicks in the assembled DNA.

Briefly, the vector pK18mobsacB, resistant to Kanamycin (Schafer et al., 1994), was digested with *Eco*RI to linearise it and then was purified using Qiagen and its concentration was measured using nanodrop (Thermo Fisher Scientific).

Primers were designed to amplify 700 bp fragments prior and after the gene to be knocked out and that were then used for the assembly. The amplified fragments from *R. pomeroyi* and the Gm cassette from pBBR (Kovach et al., 1995) had overlapping end for the assembly.

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and concentrations were measured using a nanodrop (Thermo Fisher Scientific). For the Gibson Assembly, 5 µl of a DNA mix (containing the three amplified fragments and digested plasmid in equimolar concentrations) was added to 5 µl of Gibson's assembly mixture and incubated for 1 h at 50℃.

The assembly was transformed into *E. coli* strain competent cells following manufacturer's instructions.

Transformants were streaked on LB plates containing Kanamycin (50 µg/ml) and Gentamicin (10 µg/ml).

Colony PCR was done to check for colonies with the correct assembly.

Miniprep was done for the selected colony using miniprep kit (Qiagen) with 50 µl elution and transformed into *E. coli* s171 λ pir competent cells as this strain had the conjugative machinery to deliver the plasmid by conjugation into *R. pomeroyi*.

2.5.1.3. Conjugation

The next step was to do the conjugation between the transformed *E. coli* S17-1 containing plasmid constructs and the wild type *R. pomeroyi* strain to get the mutants. 10 ml of marine broth (Difco) was inoculated with *R. pomeroyi* and incubated at 30°C in a shaking incubator for two days. *E. coli* strain containing the plasmid constructs were inoculated in 3 ml of LB broth (no antibiotics) and incubated overnight at 30°C in a shaking incubator. Then both cultures were mixed together and centrifuged at 3220 × g for 15 min at 4°C. The supernatant was discarded and the pelleted cells were resuspended and combined in the remaining supernatant. The cells were pipetted onto a marine broth (MB) agar plate in the centre and the drop of cells allowed to dry under sterile conditions. The MB agar plates with the conjugation reaction were incubated at 28°C overnight. Cells were then resuspended in 1 ml of minimal marine media (MMM) and were plated on MMM plates (table 7.2) amended with gentamicin (i.e. for the selection of *R. pomeroyi* transconjugants and recombinants) and monomethyl amine (MMA) as the only source of nitrogen (i.e. to counter select for *E. coli* cells as this strain cannot utilise this compound). Succinate was used as the source of carbon and energy.

2.5.1.4 Checking for double crossover mutants in R. pomeroyi

Transconjugants of *R. pomeroyi* were screened for double recombination. For this, all transconjugants were streaked onto an MB agar plate amended with gentamicin to a 10 µg/ml concentration and a MB agar plate amended with the antibiotic to which the vector backbone conferred resistance (i.e. ampicillin to a concentration of 100 µg/ml for those constructs in pGP704 and kanamycin 50 µg/ml for those in pK18mobsacKm). Mutant *R. pomeroyi* were

further confirmed by colony PCR and sequenced. Mutants were preserved in 20% glycerol in -80°C.

2.5.2. Experimental set up of Synechococcus co-cultures with R. pomeroyi mutants

Marine *Synechococcus* sp. WH7803 and mutants of *R. pomeroyi* were grown in optimal condition and media as described in section 2.1.1. Wild type and mutant *R. pomeroyi* cells were harvested via centrifugation and washed twice in filter-sterilized autoclaved seawater (SW, natural seawater collected from the Gulf Stream in the Gulf of Mexico; provided by Sigma, USA) prior to co-inoculating. ASW *Synechococcus* cells were also washed twice in SW for SW co-culture experiment. *R. pomeroyi* mutants and wild type *R. pomeroyi* strain were inoculated in 30 ml of ASW and SW media at final cell concentrations of 10^{7-8} and 10^{5-6} cell ml^{-1}, respectively. *Synechococcus* was added at 10^{7-8} and 10^{4-5} cell ml^{-1}, respectively. The cultures were incubated for up to 100 days in optimal conditions for *Synechococcus* as described in section 2.1.1. The axenic cultures of *Synechococcus* were also incubated for comparison. The triplicate flasks from time points i.e days 0, 3, 7, 14, 21, 37, 63, 78 and 100 were analysed. At each time point, *Synechococcus* cell abundance was monitored by flow cytometry (BD FACScan), while viable heterotrophs were counted by colony forming units on marine agar (Difco, France) as previously suggested (Christie-Oleza *et al*., 2017).

2.5.3. Experimental set up to test for substrate degradation of mutant 1.

Mutant in protein 1 (presumably a pectate hydrolase) was tested with different polysaccharides (pectin, starch, cellulose, laminarin and alginate). For this, wild type *R. pomeroyi* and pectate hydrolase mutant (AAV93776.1) were grown in 1 ml of MB media for 3 days. Cells were washed in SW and inoculated in sterile natural oligotrophic SW for 2 days to starve the cells. After 2 days, 3 µl of cells of pectate hydrolase mutant and of wild type *R. pomeroyi* were inoculated in 3 ml of ASW *pom* media (ASW *pom* media as mentioned in table 7.3 with succinate, without succinate, laminarin, pectin, starch, cellulose and alginate). The

concentration used for these different substrates was 0.1%. They were allowed to grow for 3 days. Viable cells were counted by CFU on marine agar plates.

2.5.4 Experimental set up to test for substrate degradation of mutant 3.

Mutant in protein 3 (presumably a sialidase enzyme) was tested with the monomer NANA (n-acetyl neuramic acid). For this, wild type *R. pomeroyi* and mutant in the sialidase gene (AAV95476.1) were grown in 1 ml of MB media for 3 days. Cells were washed in SW and inoculated in SW for 2 days to starve the cells. After 2 days, 3 µl of cells of the sialidase mutant and of wild type *R. pomeroyi* were inoculated in 3 ml of ASW *pom* media (ASW *pom* media with succinate, without succinate, and NANA). The concentration used for NANA was 0.1%. They were allowed to grow for 3 days. Viable cells were counted by CFU on marine agar plates.

Chapter 3

100 Days of marine *Synechococcus–Ruegeria pomeroyi* interaction: A detailed analysis of the exoproteome

Most of the information included in this chapter has been published in: Kaur, A., Hernandez-Fernaud, J. R., Aguilo-Ferretjans, M. D. M., Wellington, E. M. and Christie-Oleza, J. A. (2018). 100 Days of marine *Synechococcus-Ruegeria pomeroyi* interaction: A detailed analysis of the exoproteome. *Environmental Microbiology*. 20: 785-799.

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3.1 Introduction

Marine picocyanobacteria, mainly belonging to the genera *Prochlorococcus* and *Synechococcus*, are numerically the world's dominant photosynthetic primary producers and a major component of marine phytoplankton. Despite being outnumbered by the heterotrophic community, these cyanobacteria are responsible for half of the marine primary production and play a key role in sustaining marine food webs (Falkowski, 2012). Hence, phytoplankton are at the base of the marine food chain feeding the ecosystem with DOM and POM that is released via cell lysis (e.g., cell death, inefficient grazing and viral lysis) or other cellular processes (e.g., outer membrane vesicles, active efflux processes or permeable membrane leakage) (Biller et al., 2014; Christie-Oleza et al., 2015a; Grossowicz et al., 2017). Most of this organic matter will be used by the heterotrophic bacterioplankton as their main source of carbon and energy, returning inorganic nutrients to the phototrophic community (Christie-Oleza et al., 2017). The Roseobacter group of the class Alphaproteobacteria is an important component of the microbial community within the marine euphotic layer, accounting for up to 20% of the sea surface bacterioplankton (Wagner-Dobler and Biebl, 2006). This group of heterotrophs generally shows a positive correlation with the phytoplankton community and forms intimate associations with specific phytoplankton groups (Giebel et al., 2011; Morris et al., 2012). Interestingly, marine Roseobacter strains present a large genomic capability and metabolic versatility to use an array of organic substrates found within phytoplankton exudates and, hence, they are one of the first bacterioplankton groups to react to the input of organic matter produced, for example, during phytoplankton blooms (Newton et al., 2010; Romera-Castillo et al., 2011; Christie-Oleza et al., 2012a; Buchan et al., 2014; Landa et al., 2016; Simon et al., 2017). The strain *Ruegeria pomeroyi* DSS-3 was the first roseobacterium to have its genome sequenced (Moran et al., 2004) and has served as a model organism to study biogeochemical, ecological and physiological strategies of this group of heterotrophic marine bacteria (Christie-Oleza and Armengaud, 2015).

Around 70% of the DOM in the oceans is considered to be low-molecular weight (< 1 kDa) (Benner, 2002). The other 30% of DOM is of high molecular weight (> 1 kDa) and, curiously, is much less refractory and, hence, more readily degraded than the low-molecular weight fraction (Decho and Gutierrez, 2017). Most of the high molecular weight DOM is in the form of biopolymers, and because biological membrane systems are only permeable to molecules smaller than 0.6 kDa (Weiss et al., 1991), exoenzymes or ectoenzymes play a key pivotal role in polymeric DOM hydrolysis and assimilation (Vetter and Deming, 1999) as these biopolymers must be hydrolyzed outside the cell before they can be taken up by most organisms (i.e., non-grazing organisms). The activities of secreted enzymes in marine microbes has generally been assessed through the use of fluorescently labelled substrates (Karner and Herndl, 1992; Martinez et al., 1996; D'Ambrosio et al., 2014; Arnosti, 2015), simple observation of bacterial growth on different polymeric substrates (e.g., Mitulla et al., 2016) or, more rarely, identifying and characterizing the actual enzymes involved in hydrolyzing the DOM (e.g., Hehemann et al., 2014; Xing et al., 2015). Interestingly, marine bacterial exoenzymes are proving highly distinct from their well-characterized terrestrial counterparts (Michel and Czjzek, 2013) and are currently poorly identified.

The use of shotgun proteomics has become a powerful tool for detecting the array of proteins present in the extracellular medium of an organism under different experimental conditions (Armengaud et al., 2012) and, hence, a reliable high throughput method for identifying key secreted enzymes and other proteins involved in cell-to-cell and cell–environment interactions. Previous analysis of the exoproteome of various Roseobacter strains grown in rich media showed a diversity of trophic strategies within this clade, that is, through the abundant detection of secreted nutrient transporters, mobility proteins, adhesion-like proteins or toxins (Christie-Oleza et al., 2012b).

Nevertheless, Roseobacter strains induced a completely different array of secreted proteins when grown in the presence of a more realistic source of organic matter, that is, DOM and POM produced by marine *Synechococcus*, with an increase in proteins involved in motility,

microbial interactions, hydrolytic activities and capsid proteins of the genetic transfer agent (GTA) encoded in the genome and a strong decrease in the secretion of toxin-like proteins (Christie-Oleza et al., 2015b).

Synechococcus species generate large amounts of organic matter and require the presence of a specialized heterotrophic community to remineralise the leaked photosynthate and obtain a constant feed-back of inorganic nutrients (Christie-Oleza et al., 2017). Based on this basic principle of phototroph–heterotroph interaction, illuminated *R. pomeroyi–Synechococcus* sp. co-cultures are able to survive for extended time periods both in mineral enriched media and natural oligotrophic seawater (Christie-Oleza et al., 2017). In this study, for the first time the exoproteome of *R. pomeroyi–Synechococcus* co-cultures was analysed overr an extended time period (i.e., 100 days) both in nutrient-enriched and natural oligotrophic seawater in order to generate a unique time course dataset that would highlight the molecular mechanisms involved in this dependent microbial interaction over time. The main aims were to (i) determine protein pattern shifts over the 100-day time course and observe whether culture stability is reflected by a consistent exoproteome; (ii) identify culture stages and frame the trophic strategy of each microbe at each time point; and (iii) identify the secreted hydrolytic enzymes produced by the heterotroph and evaluate their variations over time.

3.2 Results and discussion

3.2.1 Correlation between culture growth and exoproteomes

In both nutrient-rich and oligotrophic media, the sustained survival of the co-culture comes as a consequence of nutrient cycling between the phototroph and heterotroph (Christie-Oleza et al., 2017). Here, the growth of *Synechococcus* sp. WH7803 and *R. pomeroyi* DSS-3 during the 100 days co-culture in both natural oligotrophic seawater (SW) and enriched artificial seawater (ASW) (Fig. 3.2.1.1) showed the expected trends as previously reported (Christie-Oleza et al., 2017). While *Synechococcus* sp. WH7803 reaches high cell densities in ASW (> 10^9 cells ml^{-1}) and growth is limited by light, in natural SW the cyanobacterium is limited by the availability of inorganic nutrients and cell densities reached 10^5 cells ml^{-1}, numbers that are

similar to those observed in natural marine ecosystems (Parsons et al., 2012). The heterotroph, *R. pomeroyi* DSS-3, is limited by the availability of organic carbon and vitamins regenerating essential nutrients for the phototroph. *Synechococcus–R. pomeroyi* co-cultures can persist over 200 days (Christie-Oleza et al., 2017), but a stable state is reached after just 21 days in natural SW (Fig. 3.2.1.1). However, in rich ASW medium, *Synechococcus* sp. WH7803 only enters the stable state after 100 days when cell densities drop 10-fold and stabilise at 10^8 cells ml^{-1} (Christie-Oleza et al., 2017).

Exoproteomes tend to reflect microbial adaptive strategies (Christie-Oleza et al., 2012b), and as expected, the variations observed in the exoproteomes presented here corresponded with the different growth-phase physiologies observed in SW and ASW grown co-cultures as shown by the principal component analyses (PCA) using the normalized exoproteomic data obtained from *Synechococcus* sp. WH7803 and *R. pomeroyi* over the time course experiment. The sum of the two first principal components represented 82% and 93% of the variability within the exoproteome of *Synechococcus* sp. WH7803 and *R. pomeroyi* over the 100 days, respectively (Fig. 3.2.1.2).

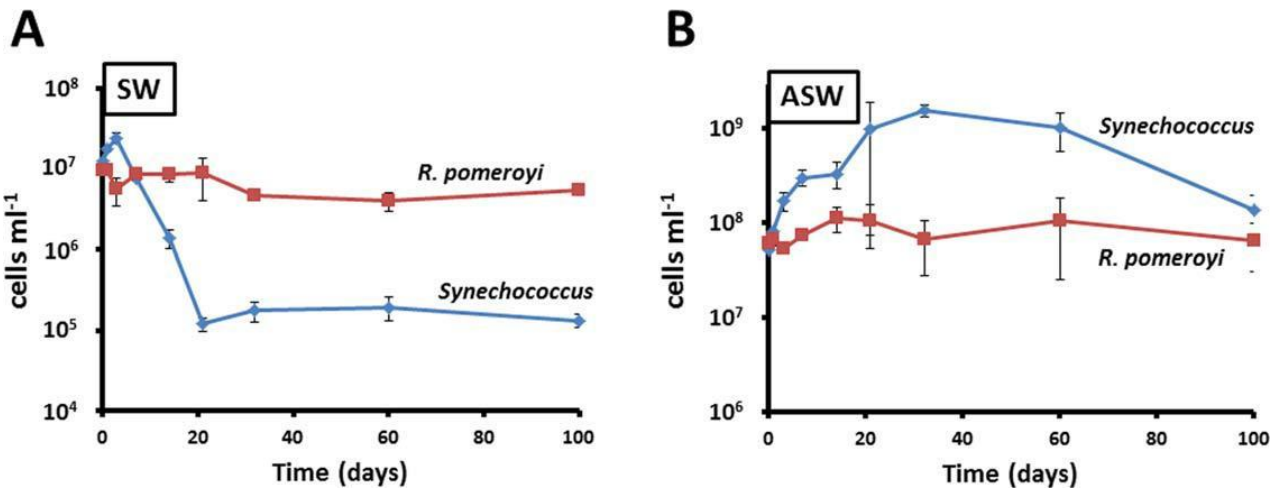

Fig. 3.2.1.1. Growth curves of *R. pomeroyi* DSS-3 and *Synechococcus* WH7803 over the 100-day time course experiment in natural SW **(A)** and ASW medium **(B)**. The average value of triplicate cultures (n = 3) is shown in panels (error bars show standard deviation).

3.2.1.1 PCA of the exoproteome of Synechococcus sp. WH7803. The PCA plots were plotted using the software PAST in which the normalised exoproteome was used. In natural SW, the exoproteome of *Synechococcus* showed a distinct shift between time points 1–14 and 21–100 days incubation (Fig. 3.2.1.2A), which coincides with the co-culture entering the stable state equilibrium observed at day 21 (Fig. 3.2.1.1). Interestingly, the exoproteome of ASW-grown *Synechococcus* sp. WH7803 remained similar over the 100 days experiment, except for time point 21 which marks the transition from the exponential to the stable state phase (Fig. 3.2.1.1). Curiously, time point 21 grouped with the stable state time points of *Synechococcus* grown in oligotrophic SW (i.e., 21–100 days; Fig. 3.2.1.2A), suggesting a transitory peak of starvation in ASW media.

3.2.1.2 PCA of the exoproteome of R. pomeroyi. The heterotroph showed a similar transition over time in both nutrient-poor and -rich media (Fig. 3.2.1.2B), mainly marked by the shift from exponential and stable state cultures. Nevertheless, the slight divergence between SW and ASW exoproteomes was mainly resolved by the principal component 2 (Fig. 3.2.1.2B), suggesting a distinctness between nutrient-rich and oligotrophic culture conditions.

Although the transitions that were observed over time are likely caused by the acclimation of the microbial populations to the varying conditions of the culture, genetic modifications (as demonstrated in Zambrano et al., 1993) cannot be ruled out and, hence, further research is needed to determine if genetic evolution also occurs during these long-term co-cultures.

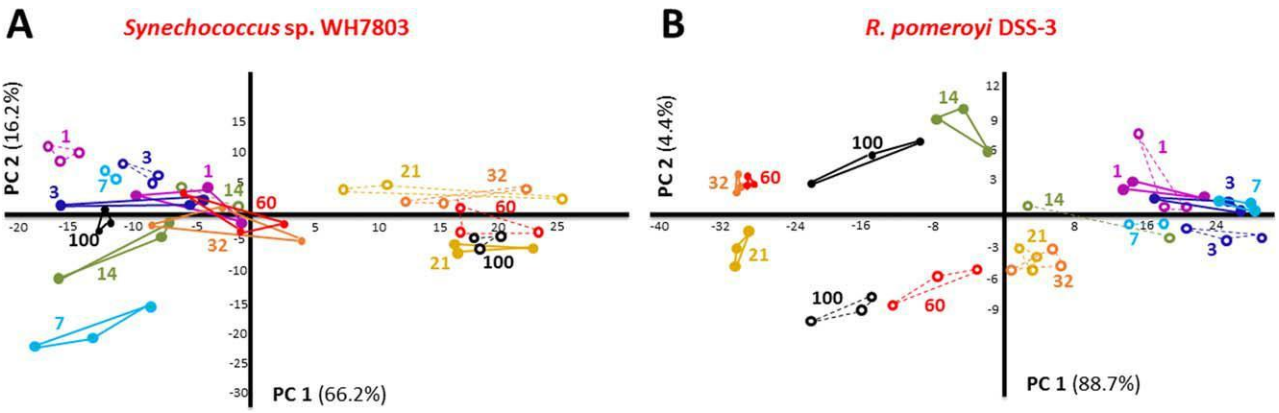

Fig. 3.2.1.2. PCA of the normalized exoproteomes of *Synechococcus* sp. WH7803 (**A**) and *R. pomeroyi* DSS-3 (**B**) when grown in co-culture in ASW medium (solid circles and lines) and natural SW (open circles and dashed lines). Numbers refer to the culture day that the samples were collected.

3.2.2 The exoproteome of Synechococcus over the 100-day time course

Just 187 and 221 of the 681 polypeptides detected in the co-culture's exoproteome belonging to *Synechococcus* already represented 95% of the total protein abundance in SW and ASW, respectively. These proteins were grouped into functional categories and represented in Figure 3.2.2.1 A.

3.2.2.1 Photosynthesis and basic cellular processes. *Synechococcus* sp. release considerable amounts of organic matter not only in the form of carbohydrates (Biersmith and Benner, 1998; Bertlisson et al., 2005) but also in the form of protein (Christie-Oleza et al., 2015a; 2017). Here, proteins involved in photosynthesis are among the most abundant categories found in the exoproteome of *Synechococcus* sp. WH7803, representing around 32% of the exoproteome (Table 3.2.2.1). Interestingly, this percentage is strikingly similar to the amount of photosynthetic proteins observed in cellular proteomic analyses (Table 3.2.2.1; Christie-Oleza et al., 2017). While this may suggest that the exoproteome of *Synechococcus* is made entirely of proteins generated from cell lysis, other indicators such as the amount of cytoplasmic or ribosomal proteins are not in agreement. While ribosomal proteins in *Synechococcus* cells represent between 5.5% and 6.5% of the total cellular proteome, only between 1.0% and 1.6% of relative abundance of these proteins are found in the extracellular

fraction (Table 3.2.2.1), suggesting that between 18% and 25% of the exoproteome may well come from cell lysis. When using cytoplasmic proteins as an indicator, the average percentage of predicted cell lysis increases to 50% (Table 3.2.2.1), but with bursts that coincide with the cell lysis expected from the growth curves. For example, the drastic reduction of *Synechococcus* cell abundance observed at days 7 and 14 under SW conditions (Fig. 3.2.2.1A) coincides with an increase in detection of cytoplasmic proteins in the exoproteome (i.e., 25–30%), suggesting that, during these time points, between 65% and 77% of the extracellular proteins may come from cell lysis. Nevertheless, at other time points cell lysis remains stably between 40% and 50%. It is interesting to note that some of these predicted cytoplasmic proteins are more abundant in the exoproteome than in the cellular fraction suggesting one of the following three possibilities: (i) they are more stable in the extracellular milieu than other cytoplasmic proteins and, therefore, over time they are enriched in the exoproteome; (ii) they might actually be actively secreted; and (iii) they are 'selectively leaked' as suggested by others (Grossowicz et al., 2017). One of these proteins is the nucleotide-binding protein SynWH7803_1823, a protein predicted to be involved in the temporal control of bacteriophage gene transcription, which represents 2.4% of *Synechococcus* exoproteome (almost 12% of the predicted cytoplasmic proteins in this fraction), whereas it only represents 0.1% in the cellular proteome. Proteins involved in photosynthesis are another example of unequal accumulation in the extracellular milieu or 'selective leakiness', and different elements of the photosynthetic apparatus are differentially partitioned, such as those from the phycobilisome (Table 3.2.2.1). Phycobilisomes should be localized on the cytoplasmic side of the thylakoidal membrane, but they seem to be enriched in the exoproteome of *Synechococcus*. Interestingly, the presence of these proteins from the photosynthetic antenna shows a strong decrease in the natural SW exoproteome over time (from 55% to 5% of the exoproteome; Fig. 3.2.2.1A), suggesting a reduction in leakiness or a decrease in the production of cellular phycobilisomes as a consequence of nutrient stress. Protein trafficking in cyanobacteria remains poorly characterized (Schneider, 2014) and the 'selective leakage'

observed here, such as of predicted cytoplasmic proteins or phycobilisomes, requires further research.

3.2.2.2 Membrane transport. The periplasmic-binding component of ABC transporters is commonly found in microbial exoproteomes (Christie-Oleza and Armengaud, 2010; Johnson-Rollings et al., 2014) and it has been suggested that some of these proteins could be intentionally translocated from the periplasm to the extracellular space (Giner-Lamia et al., 2016). The detection of membrane transporters is a good indicator of (i) the nutrients that are targeted by each organism in a community (Christie-Oleza et al., 2017) and (ii) an organisms' nutrient stress within the system (Saito et al., 2014). As expected, *Synechococcus* sp. WH7803 predominantly targets inorganic nutrients, mainly phosphate and metals such as iron. Up to three different periplasmic substrate-binding proteins for phosphate were detected in the ASW condition, although only one of them (i.e., SynWH7803_1045) was abundantly detected in both enriched and oligotrophic conditions. Interestingly, none of these periplasmic-binding proteins co-localizes in the genome with the other components of the ABC transporter for phosphate (i.e., permease and ATPase). *Synechococcus* shows a clear rise in P starvation over time under oligotrophic SW conditions as seen by the progressive increase in abundance of the periplasmic substrate binding protein for phosphate SynWH7803_1045 from 0.7% at day 1 to over 14% after the 100-day incubation. A similar trend is observed for porins that are linked to P stress (SynWH7803_0993, 0.1– 3.1%; SynWH7803_2236, below 0.1–0.7%) and an outer membrane efflux protein involved in protein secretion which needs further characterization (SynWH7803_2199, below 0.01–0.11%). In nutrient-enriched ASW media, the cyanobacterium has a peak in all nutrient transport proteins (i.e., P and metals) at day 21, which then drops until day 100 (Fig. 3.2.2.1 A), a trend that fits the variability observed in the PCA plot in Figure 3.2.1.2A.

3.2.2.3 Oxidative stress. Apart from nutrient recycling (Christie-Oleza et al., 2017), the scavenging of reactive oxygen species (ROS) is considered a pivotal process in marine cyanobacteria – heterotroph interactions where the cyanobacterium, mostly lacking of

catalase, relies on the heterotroph for depleting ROS (Hunken et al., 2008; Morris et al., 2008; 2011). Nevertheless, while catalase only catalyzes the decomposition of hydrogen peroxide, it is the enzyme superoxide dismutase (SOD) which helps deal with superoxide (O^{2-}). SODs are found in all marine cyanobacteria although with different metal cofactors (Scanlan et al., 2009). While Ni-containing SODs are prevalent in catalase-lacking strains, Fe-containing SODs prevail in catalase encoding cyanobacteria. Cellular proteomes of *Synechococcus* sp. WH7803 generally show below 1% abundance of this enzyme (Christie-Oleza et al., 2017), but SOD has also been detected in the exoproteomes of different marine *Synechococcus* strains ranging between 0.7% and 3.4% (Christie-Oleza et al., 2015a). SODs are possibly periplasmic-located enzymes with a non-canonical secretion system, but may also be 'unavoidably leaked' through the outer membrane, reinforcing the idea that ROS scavenging can be a public goods process as suggested by others (Morris et al., 2012). In this study, the abundance of the Fe-containing SOD in the exoproteome of *Synechococcus* sp. WH7803 was surprisingly high throughout the entire time course experiment both in SW and ASW averaging 24% and 19% of total protein abundance, respectively, and highlighting the relevance of this enzyme for dealing with ROS. The fact it was detected in both nutrient conditions is an indicator that its abundance is not an artefactual result and its variable abundance in ASW suggests it does not come as a consequence of its accumulation in the milieu. The second SOD encoded by *Synechococcus* sp. WH7803, the copper-containing protein SynWH7803_0951, was also detected in both SW and ASW exoproteomes with an average detection of 0.19% and 0.03%, respectively.

3.2.2.4 Other extracellular processes. The variable abundance of structural type IV pili proteins in the exoproteome of *Synechococcus* sp. WH7803 is the most remarkable aspect within this functional category. In a previous study, there was already reported a high abundance of the pili proteins SynWH7803_1795 and SynWH7803_1796 (17.9% and 1.3%, respectively), which was exclusive to this strain (Christie-Oleza et al., 2015a). The time course experiment has allowed us to assign the production of these proteins to nutrient-rich conditions

with a peak of SynWH7803_1795 detection at day 7 (25.7%) and an average detection of 8.1% over the 100 days, whereas the detection was low (< 0.1%) in natural SW conditions. The remarkable ecological and physiological roles of the pili need further characterization although a recent study in *Synechococcus elongatus* suggests this structure may be involved in cell buoyancy (Nagar et al., 2017). Interestingly, both alkaline phosphatases were detected in the exoproteome of *Synechococcus* sp. WH7803 but only in low abundance and peaked during day 21 in ASW.

3.2.2.5 Secreted proteins of unknown function. Proteins of unknown function tend to predominate in the secreted fraction, highlighting the poor knowledge we currently have on microbe–environment interactions (Christie-Oleza et al., 2015a). Datasets such as the one which are presented here are useful for flagging proteins of unknown function that are abundant and, hence, may have an important role and also to shed light on their possible function by monitoring their abundance over time. Interestingly, the five most abundant proteins in this category were common in both SW and ASW conditions, but these showed interesting shifts in abundance over time (Fig. 3.2.2.2). The conserved proteins in bacteria or marine cyanobacteria SynWH7803_0982, SynWH7803_2169 and SynWH7803_1017 showed very similar behaviour, with a progressive increase in oligotrophic SW and a peak in abundance at day 21 in ASW (Fig. 3.2.2.2), a time point when *Synechococcus* sp. WH7803 was nutrient-starved as highlighted above. Hence, these proteins may have a role in response to nutrient stress. In contrast, protein SynWH7803_1824, which is unique to strain WH7803 and conserved only among *Burkholderia*, remained at a constant low abundance but became highly abundant in SW over time (Fig. 3.2.2.2). Finally, protein SynWH7803_1556 showed identical behaviour in both culture conditions over time but with a lag of a few days in ASW.

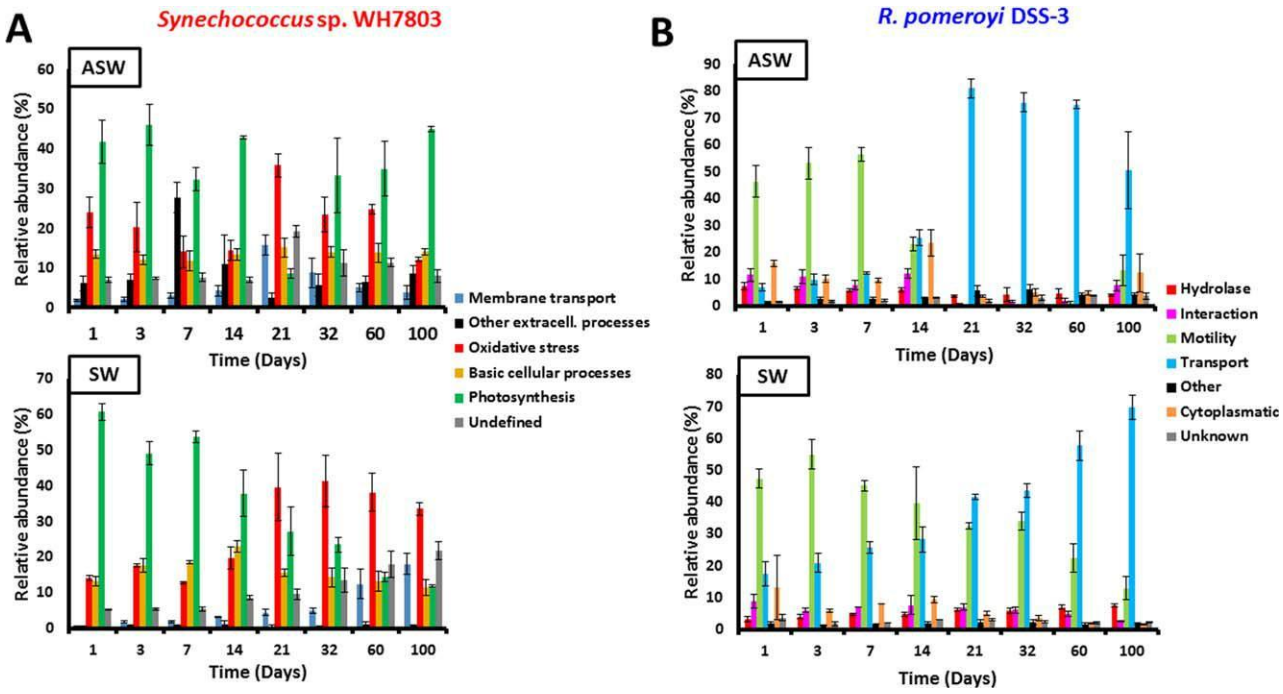

Fig. 3.2.2.1. Functional category abundance of protein found in the exoproteomes of *Synechococcus* sp. WH7803 **(A)** and *R. pomeroyi* DSS-3 **(B)** when grown in co-culture in ASW medium and natural SW. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

Table 3.2.2.1. Relative abundance of ribosomal proteins and proteins from the photosynthetic apparatus detected in *Synechococcus* sp. WH7803 proteome datasets.

	Cellular fraction[a]		Exoproteome fraction[b]			
	SW	ASW	SW		ASW	
Synechococcus						
Cytoplasmic proteins[c]	38.5 ±1.1	33.1 ±1.0	19.4 ±4.9	50%	16.6 ±2.1	50%
Ribosomal proteins	6.5 ±0.9	5.5 ±1.0	1.6 ±0.8	25%	1.0 ±0.6	18%
Photosynthetic apparatus	35.9 ±1.3	39.4 ±1.8	31.1 ±19.8	87%	32.2 ±11.6	82%
Phycobilisomes	29.8 ±1.1	28.4 ±1.6	28.2 ±19.2	95%	29.2 ±11.7	103%
Other elements	6.1 ±0.4	11.1 ±0.3	2.9 ±0.8	48%	2.9 ±0.7	26%
R. pomeroyi						
Cytoplasmic proteins[c]	57.6 ±1.3	n.a.	6.0 ±2.5	10%	10.4 ±6.4	18%
Ribosomal proteins	8.0 ±0.4	n.a.	0.2 ±0.2	3%	0.8 ±0.7	10%

n.a., not applicable due to the low number of proteins detected for *R. pomeroyi* in this analysis.

a. Data obtained from cellular proteomes published in Christie-Oleza and colleagues (2017). Standard deviation from triplicate samples is shown.

b. This study. Standard deviation from triplicate experiments from all eight sampled time points is shown. Percentages were calculated by dividing the protein relative abundance obtained from exoproteome analyses by those obtained in cellular fractions.
c. Cytoplasmic proteins were predicted using the prediction server PSORTb.

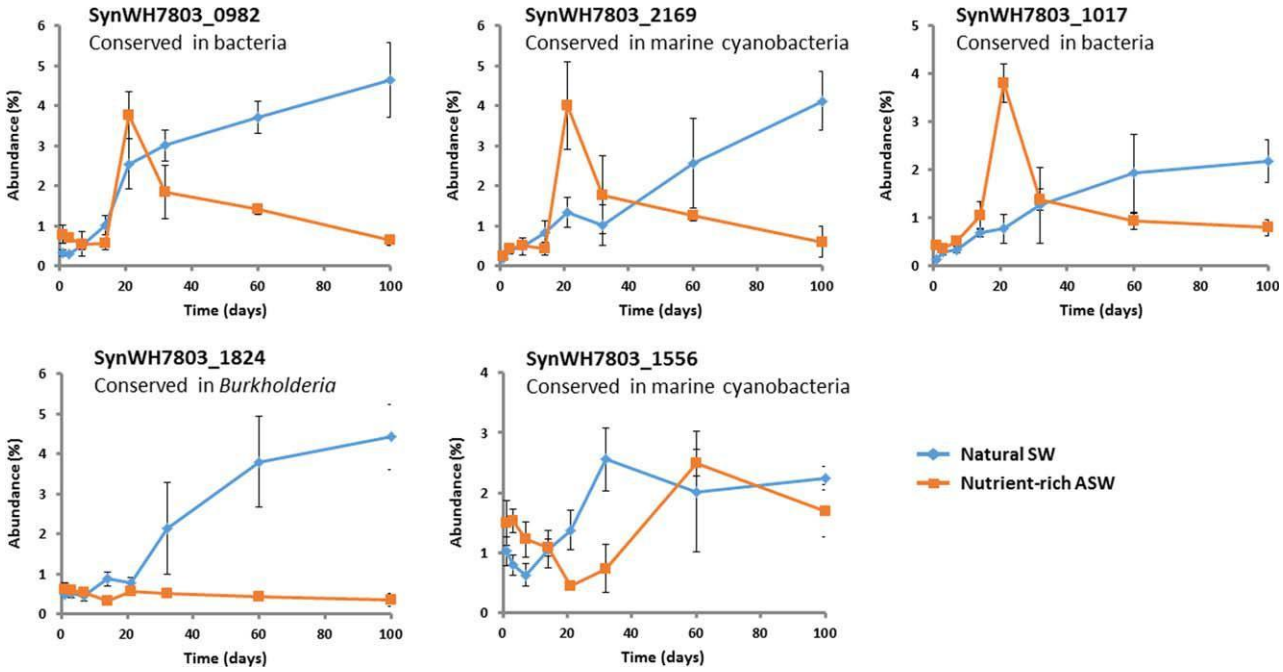

Fig. 3.2.2.2. Variation of the five most abundant hypothetical proteins in the exoproteome of *Synechococcus* sp. WH7803 over time. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

3.2.3 The exoproteome of R. pomeroyi over time

From the 585 proteins detected in the exoproteome of the co-culture belonging to *R. pomeroyi*, 184 and 222 represented over 95% of the total abundance in SW and ASW, respectively, and were grouped into functional categories and represented in Figure 3.2.2.1B.

3.2.3.1 Cytoplasmic proteins. As mentioned earlier, the detection of predicted cytoplasmic proteins and ribosomal proteins in the exoproteomes can be used as indicators of cell lysis. While the abundance of ribosomal proteins in cellular fractions of *R. pomeroyi* usually represents over 8% of the total proteome (Table 3.2.2.1), here only 0.2% and 0.8% of these proteins in the exoproteomes of SW and ASW co-cultures were observed, respectively, suggesting that between 3% and 10% of the proteins detected in this study may come from cell lysis. Nevertheless, a higher cell lysis is suggested when predicted cytoplasmic proteins is used as an indicator (i.e., 10–18%; Table 3.2.2.1). There are several reasons that could explain this discrepancy: (i) that dying cells reduce their ribosomal protein content; (ii) the size of ribosomal complexes makes them less ‘leaky’ than other smaller cytoplasmic proteins; and

(iii) the prediction of protein trafficking or selective leakiness has been under-studied. For example, under both conditions (i.e., SW and ASW), the five most abundant cytoplasmic proteins in the exoproteome of *R. pomeroyi* already contributed to over 50% of the cytoplasmic category, being three of them common between both conditions (i.e., isocitrate dehydrogenase, a hypothetical protein with high similarity to a phosphoenolpyruvate mutase and imidazoleglycerol-phosphate dehydratase), hinting that these proteins, either moonlight (Jeffery, 2009), are dually secreted at least to the periplasm, or are highly stable in the extracellular milieu and accumulate over time.

3.2.3.2 Motility. *Ruegeria pomeroyi* is a flagellum-propelled motile bacterium. The extracellular flagellin filament and hook are rarely found in the cellular proteome as they are easily sheared off during sample processing and remain in the exoproteomic fraction. The flagellin was detected in very high abundance in the exoproteomes of both SW and ASW conditions (35% and 23%, respectively). During initial culture stages flagellin represented over 50% of the total protein abundance, but while its detection only reduced progressively over time in natural SW (down to 13% at day 100), it dropped to under 1% in ASW between time points 21 and 60 days to finally increase to 13% at day 100 (Fig. 3.2.2.1 B). The sharp drop in motility between time points 21 and 60 days in ASW coincides with the maximum *Synechococcus* cell densities (Fig. 3.2.1.1) and, hence, highest photosynthate production and availability. Very low levels of flagellin were also detected in previous studies where *R. pomeroyi* was grown in carbon-rich media (e.g., the flagellin was not detected in the exoproteome of this strain when grown in marine broth; Christie-Oleza and Armengaud, 2010; Christie-Oleza et al., 2012b), highlighting the ability of *R. pomeroyi* to switch between a motile–nonmotile lifestyle, which is dependent on nutrient availability. In order to test this hypothesis, *R. pomeroyi's* motility was visualized under different nutrient conditions. No cell motility was observed when this strain was incubated in nutrient-rich marine broth (containing 0.6%, wt/vol, of organic carbon, of which 0.5% is peptone and 0.1% yeast extract) or in carbon depleted media (i.e., ASW or ASW supplemented with ammonium and vitamins). Interestingly, *R.*

pomeroyi did show notorious motility when incubated in filter-sterilized ASW media obtained from a 2-week old *Synechococcus* culture, which was predicted to contain 0.02% (wt/vol) of organic carbon (i.e., mainly protein) based on previous measurements (Christie-Oleza et al., 2017). Then the motility of *R. pomeroyi* was tested in the presence of varying concentrations of four different sources of organic matter (Fig. 3.2.3.2.1). Glucose, yeast extract and peptone induced cell motility at concentrations as low as 0.005% (wt/vol). In contrast, higher substrate concentrations of yeast extract and peptone (i.e., 0.1%) caused a drop in motility (Fig. 3.2.3.2.1), suggesting a substrate repression in flagella biosynthesis as reported previously in other heterotrophic bacteria (Stella et al., 2008). These results are in agreement with the proteomic detection of flagellin when *R. pomeroyi* was grown in the presence of *Synechococcus* and demonstrates an adaptive life strategy of this strain in response to different levels of organic matter. Hence, *R. pomeroyi* remains immotile in total absence of a carbon source, and very low concentrations of organic matter are enough to switch on its motile phenotype. The drop in flagella abundance observed in natural SW incubations (Fig. 3.2.2.1) is possibly caused by the lower concentrations of organic carbon produced by the phototroph under such nutrient-deplete conditions. Previous measurements of organic matter in such oligotrophic conditions suggest concentrations as low as 0.0002% (wt/vol). While such low substrate concentrations were not tested in these controlled experiments (Fig. 3.2.3.2.1), these small amounts of substrate seem to be enough to induce a low level of flagella production and, hence, motility in *R. pomeroyi*. This is also supported by previous observations of 5.0–6.5% flagellin detected in an exoproteomic analysis where this heterotroph was incubated in natural coastal seawater (Christie-Oleza et al 2012b). The abrupt decrease in flagellin abundance observed in nutrient-enriched ASW conditions (Fig. 3.2.2.1) is more likely caused by elevated organic matter concentrations (i.e., > 0.1%), which represses the biosynthesis of this structure. This may well simulate a phytoplankton bloom where the heterotroph turns off its motility in order to remain within the high concentration patch.

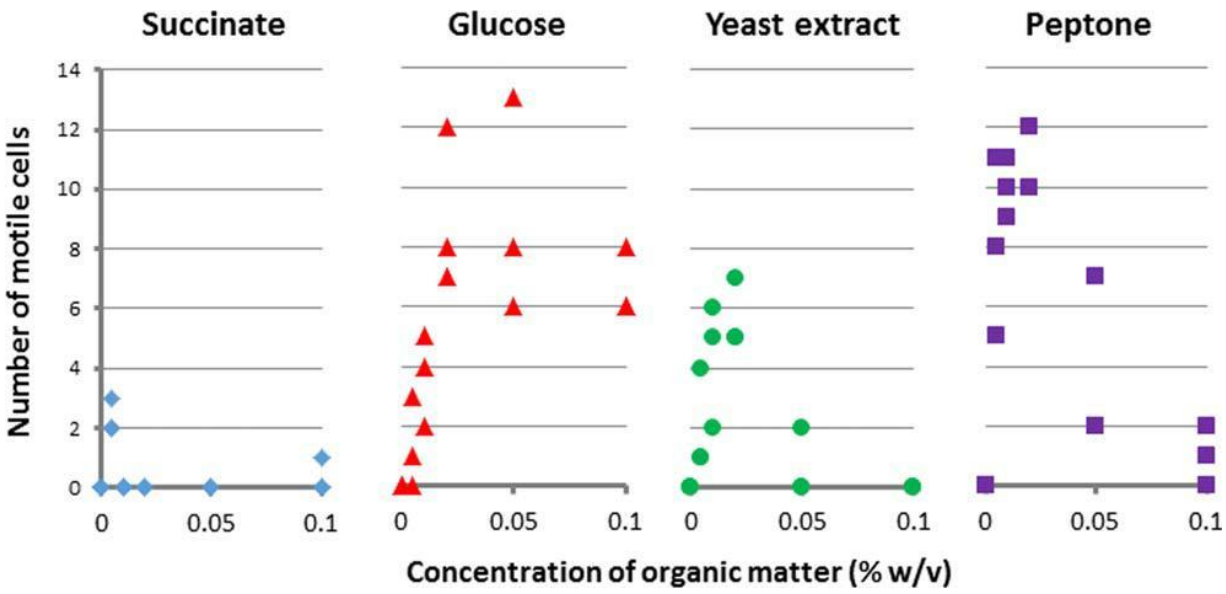

Fig. 3.2.3.2.1. Motility of *R. pomeroyi* DSS-3 in the presence of varying concentrations of different organic carbon sources. Quantification of motility was based on the number of moving cells observed in 10-s videos obtained from three different fields per condition.

3.2.3.3 Membrane transport. This is the most abundant category of proteins detected in the exoproteomes of *R. pomeroyi* (42% and 38% in ASW and SW, respectively), mostly made up of outer membrane transport systems and the periplasmic components of inner membrane transporters. For example, the periplasmic substrate-binding component of ABC transporters made up 100% of the proteins detected for these transport systems, whereas the other components (i.e., permeases and ATP-binding proteins) remained undetected. It is interesting to note that the abundance of membrane transporters mirrors the one of cell motility and highlights how the cell invests resources in response to nutrient availability (Fig. 3.2.2.1B). This generalist marine heterotroph is adapted to scavenge a large variety of scarce nutrients in oligotrophic marine systems and to proliferate rapidly in nutrient-rich patches of the ocean (Moran et al., 2004; Newton et al., 2010; Christie-Oleza et al., 2012a, b). The data we present here suggest that this life strategy is achieved by splitting resources between motility to search for nutrients when these are scarce and to rapidly take them up through the investment in membrane transporters when they are abundant. As expected, *R. pomeroyi* induces the production of a large array of specific membrane transporters for importing amino acids,

amines and carbohydrates in the presence of *Synechococcus*, which is not surprising as these are the main components of the cyanobacterium photosynthate (Christie-Oleza et al., 2017). Nevertheless, while all transporters showed an increase in abundance in accordance to the pattern shown by the 'transport' category in Figure 3.2.2.1B, the periplasmic component of the manganese ABC transporter showed a decrease in abundance in both SW and ASW conditions (Fig. 3.2.3.3.1). Although it is not clear which specific metal is transported by this transporter (i.e., manganese and zinc), *R. pomeroyi* clearly decreases its uptake possibly favouring the phototroph's high demand in metals for primary production.

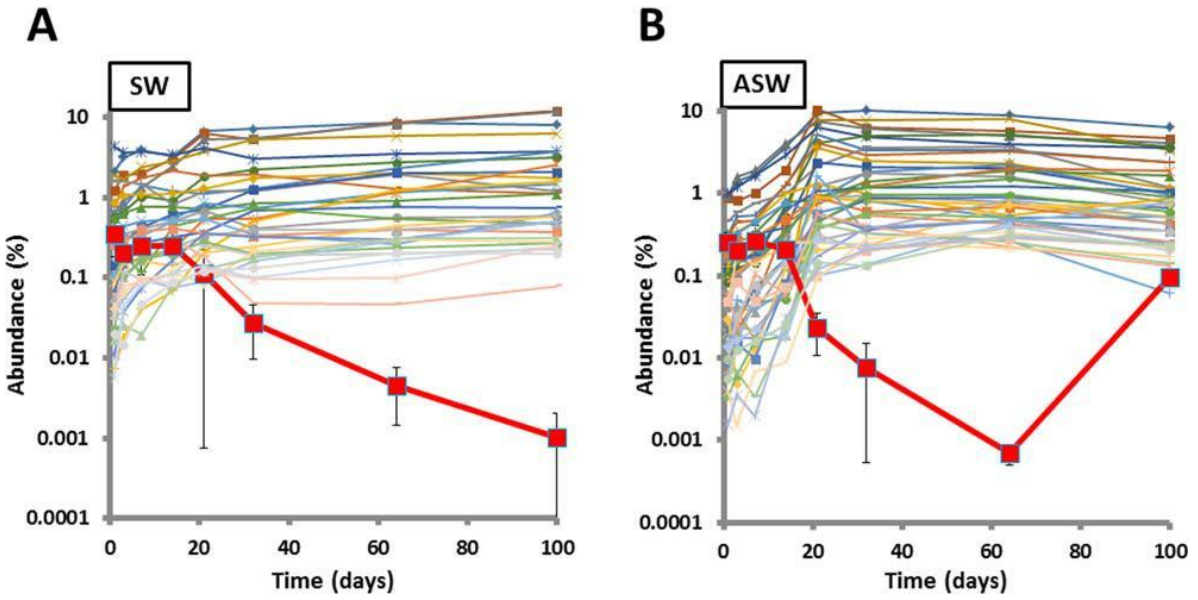

Fig. 3.2.3.3.1. Abundance of *R. pomeroyi* DSS-3 membrane transport proteins in natural SW **(A)** and ASW medium **(B)** over time. Only transporter proteins with average abundance above 0.1% are represented. The average value of triplicate cultures analyses (n = 3) are shown. The periplasmic component of the manganese ABC transporter is highlighted in bold and, for convenience, error bars showing standard deviation were added for only this protein.

3.2.3.4 Interaction. The genetic transfer agent (GTA) and Repeats-in-Toxin (RTX-like) proteins are the main contributors of this functional category. While GTAs are bacteriophage-like particles produced by a large variety of bacteria which carry random fragments from the host's genome and that are thought to encourage horizontal gene transfer (Frost et al., 2005), RTX-like proteins are elements that mainly play a direct role in cell-to-cell interactions ranging from toxicity to adhesion, although due to the enormous variability of these proteins, most are of unknown function (Linhartova et al., 2010). Interestingly, while RTX-like proteins were highly

produced in organic nutrient-rich broths, for example, the RTX-like protein PaxA represented over 50% of the proteins in the exoproteome when *R. pomeroyi* was grown in marine broth (Christie-Oleza and Armengaud, 2010), this does not occur in the mineral media used in this study, where the heterotroph is constantly supplied with a smaller amount of photosynthate. In any case, RTX-like proteins were still detected throughout the 100-day experiment (1.7% and 0.5% in ASW and SW, respectively). GTA are commonly found in Roseobacter strains (Biers et al., 2008; Zhao et al., 2009). The high production of GTA by *R. pomeroyi* when grown in co-culture with *Synechococcus* was highlighted previously (Christie-Oleza et al., 2015b). However, from the time course data, it is interesting to note that the abundance of GTA elements (with an average protein abundance of 4.6% and 5.7% in ASW and SW, respectively) mimics that of the flagella, that is, dropping from 5% to 9% during days 1–14, to between 0.1% and 0.3% during time points 21–64 days in ASW. This suggest a co-regulation between the motile 'scavenging-for-nutrients' lifestyle mode and the burst of GTA production, possibly induced by the sensing of low nutrient availability. In fact, GTA production is known to be inhibited by high concentrations of phosphate (Westbye et al., 2013).

3.2.3.5 Hydrolytic enzymes for polymeric organic matter. One of the aims of this study was to highlight and monitor the production of potential secreted enzymes involved in the breakdown of phototrophic DOM over time. Marine Alphaproteobacteria are considered to have a very low extracellular hydrolytic potential due to the low number of enzymes that are encoded in their genomes (Barbeyron et al., 2016). In fact, previous exoproteomic analyses from a range of Roseobacter strains showed that different isolates produced a small but highly divergent, repertoire of exoenzymes suggesting that closely related species may be able to target different substrates and escape competition through the diversification of resources (Christie-Oleza et al., 2015b). Here, the overall average abundance of this category was 5.3% and 5.5% for ASW and SW, respectively, but while the abundance of these proteins progressively dropped in ASW (7.4% at day 1 to 4.1% at day 100), an opposite trend was observed in SW with an increase from 3.3% to 7.6% during the 100-day time course (Fig.

3.2.2.1B). Nineteen potential hydrolytic enzymes produced by *R. pomeroyi* were detected during this study (i.e., with an average detection > 0.1% in at least one of the culture conditions; Table 3.2.3.5.1). The two most abundantly detected hydrolases were AAV93776 (2.1% and 2.7% in ASW and SW, respectively) and AAV95476 (0.9% and 2.68% in ASW and SW, respectively), re-annotated here as a possible pectate lyase and sialidase, respectively (Table 3.2.3.5.1). Hence, both proteins may be involved in polysaccharide hydrolysis, generating oligosaccharides and short chain sugars that can then be assimilated by the heterotroph. Despite being highly abundant, the pectate lyase-like protein shows a drastic reduction in the ASW co-culture between time points 21 and 60 coinciding with the period when *Synechococcus* is most abundant (Fig. 3.2.1.1). During this time period, *Synechococcus* is possibly at its maximum production of photosynthate and *R. pomeroyi* may fill its carbon and energy demands through the use of protein and other organic nitrogen compounds. Interestingly, it is between days 21 and 60 when *R. pomeroyi* shows an increased production of proteases (i.e., AAV95890 and AAV97448) and amidohydrolases (AAV97448 and AAV94260). Members of the Roseobacter group are known to preferentially target small nitrogen-rich DOM (Bryson et al., 2016; Teira et al., 2017), and hence, it is not surprising that these organisms will specialize in using these compounds when they are present. While the abundance of organic matter in ASW allows *R. pomeroyi* to shift its targeted polymeric DOM, in SW it shows a more stable production of hydrolases throughout the 100-day co-culture, with only the increase of the pectate lyase AAV93776 (from 1.4% to 3.6%) and the protease AAV95890 (from < 0.01% to 0.13%) over time. *Ruegeria pomeroyi* encodes and produces most enzymes required to break-down the polymeric components within the cyanobacterial photosynthate (i.e., protein, polysaccharides, peptidoglycan and other sulfur and amide compounds), and hence, it is able to mineralize most of the primary produced organic matter, enabling the establishment of long-term co-cultures based on nutrient cycling described previously (Christie-Oleza et al., 2017). Nevertheless, it is acknowledged that the identification of most hydrolytic enzymes in Table 3.2.3.5.1 requires further experimentation to confirm their predicted function. For example, the most abundant enzyme detected, i.e., the pectate lyase-

like protein, was annotated as a hypothetical protein and only suggested here as a hydrolytic enzyme for polysaccharides based on a pectate lyase-like domain it contains. Other pectate lyases have been detected in marine microbes, but these seem to have been acquired from a terrestrial origin (Hehemann et al., 2017). Nevertheless, this is not the norm and most marine microbes have a novel array of untapped hydrolytic enzymes that largely differ from their well-known terrestrial counterparts (Hehemann et al., 2014), which require further characterization.

3.2.3.6 Hydrolytic enzymes for phosphate. Both alkaline phosphatases PhoX and PhoD encoded by *R. pomeroyi* were found in the exoproteomes (Table3.2.3.5.1). PhoX, and not PhoA, is prevalent in marine microbes and is thought to play an important role in marine oligotrophic systems (Sebastian and Ammerman, 2009; 2011). PhoX was the predominant alkaline phosphatase in this dataset (Table 3.2.3.5.1). Nevertheless, the less-known PhoD, which was in very low abundance throughout most of the time course (abundance below 0.06%), peaked only at time points 32 and 60 (0.43% and 0.78%, respectively) under ASW conditions. While the substrates targeted by PhoX have been characterized (Sebastian and Ammerman, 2011), organic phosphate compounds targeted by PhoD remain unknown. *Ruegeria pomeroyi* also abundantly secretes the nucleotidase AAV96145 when grown in co-culture with *Synechococcus*. It is worth noting the progressive increase in this nucleotidase especially under SW conditions (from 0.06% to 1.49%), which coincides with a strong decrease in PhoX abundance (Table 3.2.3.5.1). The ABC transporters for phosphate and organic phosphate (e.g., glycerol-3-phosphate) remain constant during the whole time course, suggesting that *R. pomeroyi's* phosphorous starvation status does not vary over time, but the switch from PhoX to a nucleotidase indicates a variation in the organic phosphorous produced by *Synechococcus*. It is hypothesized that *Synechococcus* may replace its phospholipids for sulfolipids (Van Mooy et al., 2009) and even initiate the accumulation of poly-phosphates (Martin et al., 2014), which may become, together with nucleic acids, the main source of phosphorous in the system.

Table3.2.3.5.1. Hydrolytic enzymes detected over time in the exoproteome of *R. pomeroyi* DSS-3 when co-cultured with *Synechococcus* sp. WH7803.

Locus ID	Annotation (possible function)	Substrate		Day 1[a]	Day 3[a]	Day 7[a]	Day 14[a]	Day 21[a]	Day 32[a]	Day 60[a]	Day 100[a]	Average
AAV93776	Hypothetical SPO0459 (pectate lyase)	Polysaccharides	ASW	4.94	4.05	3.23	3.08	0.04	0.02	0.08	1.63	2.13
			SW	1.43	1.88	2.16	2.29	3.08	3.12	3.84	3.61	2.68
AAV95476	BNR/Asp-box protein (sialidase)	Polysaccharides	ASW	1.14	1.00	0.99	1.35	0.80	0.43	0.51	1.07	0.91
			SW	1.07	1.08	1.31	1.22	1.77	1.59	1.49	1.25	1.35
AAV95139	Twin-arginine pathway (phosphatase PhoX)	Phosphates	ASW	0.58	0.56	0.40	0.33	0.16	0.88	0.65	0.15	0.46
			SW	0.53	0.54	0.57	0.28	0.13	0.08	0.02	< 0.01	0.27
AAV96145	Ser/Thr Phosphatase/nucleotidase (nucleotidase)	Nucleotides	ASW	0.04	0.05	0.27	0.13	0.93	0.77	0.70	0.34	0.40
			SW	0.06	0.18	0.23	0.44	0.69	0.58	1.02	1.49	0.59
AAV95890	Protease, S2 family (serine protease)	Proteins	ASW	0.03	0.01	0.01	0.02	0.51	0.56	0.55	0.09	0.22
			SW	< 0.01	0.01	0.01	0.01	0.01	0.03	0.07	0.13	0.03
AAV96272	Metallo-beta-lactamase (alkyl sulfatase)	Others	ASW	0.06	0.11	0.12	0.22	0.38	0.26	0.28	0.22	0.21
			SW	0.03	0.10	0.23	0.28	0.35	0.37	0.38	0.43	0.27
AAV93580	Alkaline phosphatase (phosphatase PhoD)	Phosphates	ASW	0.01	0.04	0.03	0.01	< 0.01	0.43	0.78	0.01	0.17
			SW	0.01	0.06	0.04	0.02	0.01	0.02	0.01	< 0.01	0.02
AAV95931	LysM domain/M23/M37 peptidase (peptidase family M23)	Proteins	ASW	0.12	0.28	0.19	0.15	0.11	0.18	0.11	0.10	0.16
			SW	0.05	0.08	0.07	0.06	0.02	0.02	0.01	0.06	0.05
AAV97448	Amidohydrolase protein (amidohydrolase)	Others	ASW	0.04	0.03	0.04	0.04	0.32	0.26	0.27	0.16	0.14
			SW	0.02	0.07	0.11	0.15	0.15	0.08	0.18	0.40	0.14
AAV95236	Beta-lactamase protein (carboxypeptidase)	Proteins	ASW	0.01	0.05	0.24	0.36	0.03	0.05	0.05	0.09	0.11
			SW	< 0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
AAV96841	Peptidoglycan-binding protein (glycoside hydrolase)	Peptidoglycan	ASW	0.06	0.13	0.07	0.11	0.06	0.10	0.12	0.04	0.09
			SW	0.02	0.09	0.07	0.06	0.03	0.01	0.01	0.03	0.04
AAV94622	Periplasmic serine protease (serine proteases)	Proteins	ASW	0.04	0.05	0.04	0.11	0.06	0.06	0.04	0.03	0.05
			SW	0.01	0.02	0.01	0.04	0.02	0.02	0.01	< 0.01	0.01
AAV95689	Fumarylacetoacetate hydrolase (aromatic hydrolase)	Others	ASW	0.02	0.02	0.05	0.04	0.09	0.07	0.06	0.03	0.05
			SW	0.02	0.03	0.03	0.03	0.02	0.03	0.06	0.05	0.03
AAV96493	Glycosyl hydrolase, family 25 (acetylmuramidase)	Peptidoglycan	ASW	0.08	0.10	0.10	< 0.01	< 0.01	< 0.01	< 0.01	0.01	0.04
			SW	0.03	< 0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	0.01
AAV94066	Cyclase family protein (aromatic hydrolase)	Others	ASW	< 0.01	< 0.01	< 0.01	< 0.01	0.07	0.07	0.06	0.04	0.03
			SW	< 0.01	< 0.01	0.01	0.01	0.01	0.02	0.04	0.07	0.02
AAV93452	Murein endopeptidase (murein endopeptidase)	Peptidoglycan	ASW	< 0.01	0.01	0.02	0.02	0.01	0.02	0.08	0.02	0.02
			SW	< 0.01	< 0.01	0.02	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.01
AAV95558	Hypothetical SPO2296 (lysozyme)	Peptidoglycan	ASW	0.02	< 0.01	0.01	0.01	0.01	0.01	0.04	0.03	0.02
			SW	0.02	< 0.01	< 0.01	< 0.01	< 0.01	0.02	< 0.01	< 0.01	0.01
AAV94260	Amidohydrolase protein (amidohydrolase)	Others	ASW	< 0.01	< 0.01	< 0.01	< 0.01	0.04	0.02	0.02	0.01	0.01
			SW	0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
AAV96597	Peptidase, M16 family (peptidase M16)	Proteins	ASW	< 0.01	< 0.01	< 0.01	< 0.01	0.03	0.03	0.02	< 0.01	0.01
			SW	< 0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

a. The values shown are the average of relative abundance in percentage obtained from three biological replicate cultures.

3.3 Conclusions

Microbial exoproteomes are good proxies to study the dynamic interactions of microbes with their environment. Here a unique time course exoproteomic dataset of a 100-day long *Synechococcus* sp. WH7803–*R. pomeroyi* DSS-3 co-culture incubated in both nutrient rich and natural oligotrophic seawater was generated. The observed protein variations matched well with the culture's physiology over time and emphasizes the need of time course experiments such as the one we present here in order to obtain a comprehensive understanding of microbial interactions, as single time points may be misleading. This study has highlighted a number of interesting aspects in this phototroph–heterotroph system such as (i) the heterotroph's varying motility lifestyle depending on nutrient availability, (ii) the unexplained selective leakage of phycobilisomes to the milieu by *Synechococcus*, (iii) the specificity of nutrient acquisition though the production of an array of active membrane transport systems, (iv) the large production of SOD by *Synechococcus* to deal with ROS despite the presence of a heterotroph, (v) the varying abundance of a type IV pili structure produced by the phototroph, (vi) a list of uncharacterized hydrolytic enzymes secreted by *R. pomeroyi* to mineralise the polymeric organic matter generated by the cyanobacterium, (vii) a pattern of phosphatases that varies over time and that is believed to adapt to the pool of organic phosphorous present in the system and (viii) a list of relevant proteins of unknown function that require further research. Hence, the high-resolution time course dataset we present here will become a reference for future characterization of specific molecular mechanisms involved in sustaining this microbial system.

Chapter 4

Analysis of the exoproteome of 16 different marine heterotrophs in the presence/absence of *Synechococcus* WH7803.

Acknowledgements: Culture set up and proteomic data generation was performed by MSc student Natacha Chenevoy. All the data was analysed by the author (Amandeep Kaur).

4.1 Introduction

Phototrophs and heterotrophs play an important role in the processing of the carbon in the ocean in which dissolved organic carbon, produced by phototrophs, is utilized by heterotrophs and the remineralisation by this group provides essential nutrients to the phototrophs (Christie-Oleza et al., 2017, Sarmento and Gasol, 2012, see Chapter 1). Long term co-culturing of *Synechococcus* WH7803 and *R. pomeroyi* DSS-3 proved that the phototroph-heterotroph interactions are based on nutrient recycling (Christie-Oleza et al., 2017) and DOM degradation (Chapter 3). Current knowledge indicates that these positive interactions are also based on vitamin exchange and alleviation of oxidative stress as suggested by the Black Queen Hypothesis (Morris et al., 2012). *Prochlorococcus,* a sister genus of *Synechococcus* lacks catalase and depends on the helper strain, i.e. heterotrophs, to decompose hydrogen peroxide (Morris et al., 2008). Other studies also indicate that phototroph-heterotroph interactions are based on vitamin exchange such as during the Roseobacter *Ruegeria pomeroyi* DSS-3 interaction with the diatom *Thalassiosira pseudonana* CCMP1335: while *R. pomeroyi* depends upon the diatom to uptake the fixed carbon, the diatom obtains the essential vitamin B12 from the heterotroph (Durham et al., 2014). *T. pseudonana* is unable to synthesize vitamin B12 and depends upon the external source for it (Armbrust et al., 2004), whereas Roseobacter strains carry the genes involved in vitamin B12 biosynthesis (Newton et al., 2010). Roseobacters are very active metabolically (Suzuki et al., 2001) and are detected to be associated with a variety of marine phototrophic organisms (Amin et al., 2012). Some interactions have already been seen studied between marine algae (e.g. *Emiliania huxleyi*) and Roseobacter members (Ramanan et al., 2016).

Most phototroph-heterotroph interactions are positive. Most of the strains used in this chapter produced hydrolytic enzymes and express transporters in the periplasm driving nutrient exchange and, consequently, a positive interaction with the phototroph. *Salinispora tropica* is the exception to the general phototroph-heterotroph positive interactions that are usually observed. The Actinomycete *S. tropica* kills the phototroph, as detected in the Christie-Oleza

lab in which this strain showed strong negative interactions with the phototrophs *Synechococcus* sp. WH7803 and *Emiliania huxleyi* strain under nutrient rich conditions, possibly through the production of some antimicrobial agent (Despoina Sousoni Doctoral Thesis). The CFB group has also a specialised role to uptake and degrade the DOM (Kirchman, 2002). Usually, high molecular weight polysaccharides are degraded to small molecules by extracellular enzymes of marine bacteria, and sugar monomers and oligomers are then available to surrounding microbial community. Recently, though, an alternative mechanism has been detected in the CFB group of marine bacteria that can utilize these polysaccharides in a different way (Reintjes et al., 2017). Figure 4.1.1 illustrates how polysaccharides can be up taken and hydrolysed within the periplasm of the cell. This is actually to be considered as a selfish mechanism in which when polysaccharides are attached to the outer membrane, they get partially hydrolysed and are transported to periplasm as larger oligosaccharides through TonB dependent outer membrane receptors. In the periplasm, these large oligosaccharides are further hydrolysed into monosaccharides which are then transported to the cytoplasm (Reintjes et al., 2017). Actually, this mechanism helps to reduce the loss of hydrolytic product into the environment and is detected in Bacteroidetes. The Roseobacter clade is a key group involved in DOM remineralisation and recycle essential nutrients as indicated by their abundance and high metabolic activity during phytoplankton blooms (Poretsky et al., 2010, Buchan et al., 2014). *Ruegeria pomeroyi* have been reported to contain an unusually large number of ABC and TRAP transporters to utilize the available nutrients (Christie-Oleza and Armenguad, 2010). *R. pomeroyi* and *Synechococcus* WH7803 long term co-culture illustrated that *R. pomeroyi* uses most of its membrane transporters to uptake organic compounds (59% versus 2.7% for inorganic compounds) and *Synechococcus* invests in acquiring inorganic compounds (85.2% versus 2.8% for organic compounds). Hence, both microorganisms target different substrates (Christie-Oleza et al., 2017). Gammaproteobacteria encode a large pool of CAZymes to degrade the phytoplankton exudates (Muhlenbruch et al., 2018) and, more specifically, algal polysaccharides (Lombard et al., 2014). During phytoplankton blooms, the abundance of CAZymes belonging to

Gammaproteobacteria increases following the bacterioplankton community succession (Sperling et al., 2017).

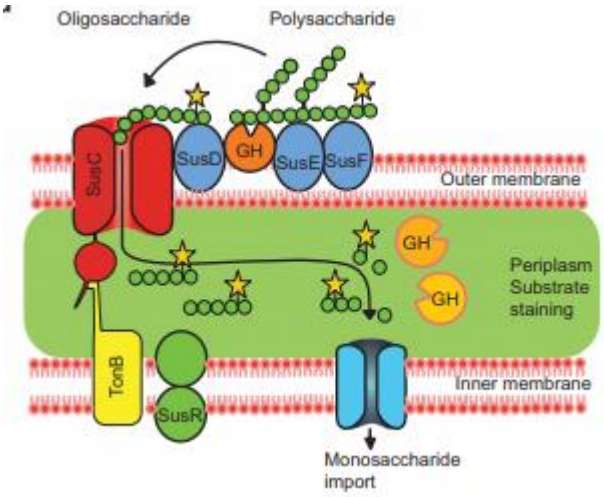

Figure 4.1.1: This figure illustrates the uptake of polysaccharides without the loss of hydrolysed product into the environment (Modified from Reintjes et al., 2017). TonB are outer membrane receptors, Sus represents starch utilization system and GH are glycoside hydrolases.

The main aim of this chapter was to investigate how marine microbes from different phylogenetic groups interact with the DOM produced by marine phototrophs and to study the relationship between photoautotrophic and heterotrophic bacteria. The life strategy of sixteen different heterotrophic bacteria when grown in the presence of the DOM produced by *Synechococcus* was analysed via a detailed exoproteomic analysis. Through the data generated we may infer a better understanding of how DOM is utilized by different relevant marine heterotrophs and how essential nutrients like nitrogen, phosphorus and trace metals are recycled (Azam et al., 1998, Pedler et al., 2014).

A collection of 16 different heterotrophs (Table 1.7.1) were grown in monoculture and in co-culture with the photoautotroph *Synechococcus* sp. WH7803 to detect possible patterns in their secreted proteins. Special attention was given to relevant exoenzymes found in higher abundance in the presence of the phototroph. The relevant method has been explained in chapter 2 of materials and methods in sections 2.1.2, 2.2, 2.3 and 2.4. The cell counts of *Synechococcus* in a co-culture with different heterotrophs from time point 0 and time point 3

have been mentioned in an appendix table 7.4. The CFU of different heterotrophs in a mono-culture and co-culture with *Synechococcus* at time point 0 and time point 3 is given in an appendix table 7.5. The features of these different heterotrophs have been mentioned in table 1.7.2 of chapter 1.

4.2 Results and discussion

The relative abundance (%) of all the differentially produced classes of the exoproteome in mono-culture of each of the heterotroph and co-culture with *Synechococcus* has been summarised in supplementary table S1. Table S1 also represents the significant increase and decrease in co-culture.

Table S1: Table summarising the relative abundance of different classes in mono-culture and co-culture. ↑ represents significant increase and ↓ represents significant decrease in co-culture with *Synechococcus*. No arrow means not significantly different.

Group	Name of organism	Culture condition	Hydrolase (%)	Interaction (%)	Motility (%)	ROS (%)	Transport (%)	Other (%)	Unknown (%)	Cytoplasmic (%)
Actinobacteria	*S. tropica*	Mono-culture	37.46 ↑	19.52 ↓	Not detected	6.72 ↓	15.46 ↓	0.80 ↑	4.08 ↓	14.92 ↓
		Co-culture	58.14	5.76		5.70	10.14	0.98	3.19	14.57
	A. marinum	Mono-culture	13.63 ↓	1.07 ↑	Not detected	4.01	16.71 ↓	3.85 ↑	0.94 ↑	55.77 ↑
		Co-culture	12.11	2.24		4.16	9.85	4.57	3.72	56.56
CFB group	*Polaribacter sp.*	Mono-culture	10.23 ↓	1.57 ↓	7.87 ↓	1.75	26.64 ↓	12.14 ↑	9.08 ↑	27.63 ↑
		Co-culture	9.04	1.53	4.33	2.45	22.08	13.68	10.08	33.04
	A. machipongonensis	Mono-culture	16.18 ↑	0.58 ↑	1.36	2.37	3.69	5.39 ↑	21.06 ↑	46.12 ↓
		Co-culture	17.26	0.83	1.11	3.69	3.86	5.74	27.42	36.91
	G. forsetii	Mono-culture	43.72 ↓	1.74 ↑	0.05	7.76	5.23 ↑	7.95 ↓	16.19 ↓	15.10 ↓
		Co-culture	36.47	4.48	0.26	5.19	20.88	3.95	13.24	13.09
	F. agariphila	Mono-culture	27.28 ↓	0.42	0.87	2.84	1.93 ↓	6.18 ↑	1.18 ↑	54.84 ↓
		Co-culture	22.47	0.76	0.77	3.56	1.71	7.09	2.76	53.28
Alpha proteobacteria	*R. pomeroyi*	Mono-culture	2.30 ↑	31.61 ↓	5.57	0.32	20.32 ↑	2.77 ↑	1.63 ↑	30.69 ↑
		Co-culture	4.03	6.07	7.54	0.68	32.90	3.40	2.09	36.79
	R. denitrificans	Mono-culture	1.51 ↑	11.40 ↓	18.19	0.46	15.39 ↑	4.10 ↓	39.99 ↓	7.67 ↑
		Co-culture	2.10	10.74	14.72	0.90	43.38	3.19	7.48	12.16
	D. shibae	Mono-culture	4.61 ↑	19.14 ↓	37.91 ↑	0.60 ↓	12.64 ↑	7.44 ↓	3.85 ↓	6.82 ↓
		Co-culture	4.75	4.84	62.76	0.26	15.55	3.34	1.53	4.93
Gamma proteobacteria	*P. citrea*	Mono-culture	43.74 ↑	5.71 ↑	9.43 ↓	0.38 ↓	21.43 ↓	2.26 ↑	15.50 ↓	0.85 ↓
		Co-culture	61.12	6.81	4.32	0.05	11.84	3.04	12.19	0.22
	A. macleodii	Mono-culture	24.58 ↓	12.87 ↓	38.45	0.51 ↑	3.98 ↓	7.96 ↑	8.59 ↑	1.70 ↓
		Co-culture	9.43	3.25	60.91	0.60	3.23	10.53	10.25	0.93
	M. adhaerens	Mono-culture	6.34 ↑	2.93 ↓	26.99	0.13 ↑	39.15 ↓	15.67 ↓	2.19 ↑	4.33 ↓
		Co-culture	13.64	2.57	41.24	0.16	24.80	9.46	5.15	1.03
	P. stutzeri	Mono-culture	0.82 ↑	3.53 ↓	33.04 ↑	0.53	29.24 ↑	15.60 ↓	4.32 ↓	9.85 ↓
		Co-culture	0.89	0.68	45.09	0.42	32.17	13.38	2.42	3.85
Planctomycetes	*P. limnophilus*	Mono-culture	3.29 ↓	22.45 ↓	5.67	Not detected	0.88	26.70 ↓	31.88 ↑	10.75 ↓
		Co-culture	0.82	3.74	8.86		0.94	4.13	33.31	2.10
	R. baltica	Mono-culture	0.77	41.32	49.78	Not detected	Not detected	9.39	Not detected	Not detected
		Co-culture	0.64	39.93	41.38			8.71		
Verrucomicrobia	*V. bacterium*	Mono-culture	22.24 ↓	2.88 ↓	Not detected	3.49 ↑	2.20 ↓	27.09 ↓	2.69 ↑	22.94 ↑
		Co-culture	15.90	2.23		4.21	1.12	15.52	8.10	45.49

4.2.1 Exoproteome analysis of 16 different heterotrophs

All the proteins that were detected in the exoproteome of 16 different heterotrophs when grown in mono-culture and co-culture with *Synechococcus* were grouped into different functional categories as explained below. For the purpose of the aims of this chapter, up- and down-regulated proteins in the presence of *Synechococcus*, as well as highly abundant proteins in the exoproteome of all 16 heterotrophs are discussed in detail and structured below according to the phylogeny of each one of the heterotrophic organisms.

4.2.1.1 Actinobacteria group: *S. tropica* and *A. marinum* are model marine Actinobacteria and possess important characteristics, which supports the decision to include them in this study. Members of the Actinobacteria have been reported to play an important role in the breakdown and recycling of organic compounds (Weyland et al., 1969).

4.2.1.1.1 Salinispora tropica: The total number of proteins detected in the exoproteome of *Salinispora tropica* was 145. Interestingly, the most abundantly detected category in the exoproteome of *S. tropica* were hydrolases (37% and 58% in mono- and co-culture, respectively; figure 4.2.1.1.1). The most common hydrolases were glucosidases, proteases and lipases suggesting that *S. tropica* can potentially breakdown the polysaccharides, proteins and lipids present in DOM of *Synechococcus*. ABP55599, annotated as a hypothetical protein, was highly upregulated in co-culture (123.5x fold change increase; table 4.2.1.1.1). Further bioinformatics analysis suggests it may be an SGNH or GDSL hydrolase, which are hydrolytic enzymes with a broad range of substrate specificity but, mainly, play a potential role in the hydrolysis and synthesis of ester compounds (Akoh et al., 2004). Most interestingly, an alpha amylase and alkaline phosphatase were also strongly upregulated in co-culture with a fold change of 99.9 and 13.8, respectively (table 4.2.1.1.1). It is surprising that two alpha-amylases of *S. tropica* (table 4.2.1.1.1), as well as other amylases in other heterotrophs (see below), are strongly up-regulated in the presence of *Synechococcus* when it is known that marine cyanobacteria rarely produce carbon storage polysaccharides such as starch (Scanlan et al., 2009). These amylases may by hydrolysing the lipopolysaccharide or, maybe, the sucrose that is known to be present in marine cyanobacteria (Scanlan et al., 2009). Nevertheless, more research is required to confirm this.

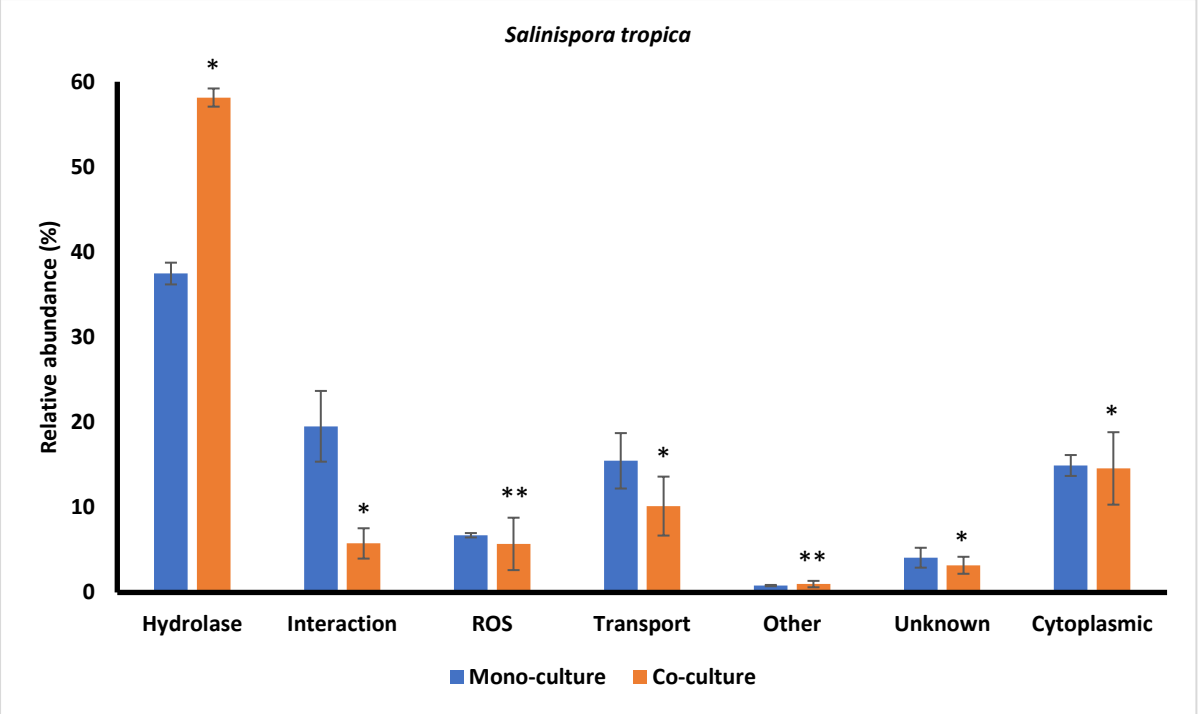

Figure 4.2.1.1.1.: Functional category abundance of proteins found in the exoproteome of *S. tropica* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.1.1: List of up and down-regulated proteins of *S. tropica* of co-culture vs mono-culture.

Accession no.	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
ABP55599	hypothetical protein Strop_3165 (SGNH_hydrolase, or GDSL_hydrolase, is a diverse family of lipases and esterases).	Hydrolase	123.5	0	0.70	Signal peptide
ABP54455	Alpha-amylase	Hydrolase	99.9	0	2.71	Signal peptide
ABP56633	hypothetical protein Strop_4205 (Domain of unknown function)	Unknown	17.4	0	0.16	Signal peptide
ABP53242	Alkaline phosphatase	Hydrolase	13.8	0	0.05	Signal peptide
ABP55130	alpha amylase, catalytic region	Hydrolase	12.1	0	6.96	Signal peptide
ABP55699	putative alpha-1,2-mannosidase	Hydrolase	-8.5	0.02	0.00	Signal peptide
ABP55766	L-glutamine synthetase	Cytoplasmic	-13.6	0	0.04	>0.1
ABP54507	catalase/peroxidase HPI	ROS	-14.0	0	0.00	-
ABP54098	Substrate-binding region of ABC-type glycine betaine transport system	Transport	-88.3	0	0.00	Signal peptide
ABP52771	phosphate binding protein	Transport	-110.1	0	0.01	Signal peptide

4.2.1.1.2 Aeromicrobium marinum: The total number of proteins detected in the exoproteome of *A. marinum* was 482. The most common hydrolases detected in the exoproteome of *A. marinum* were proteases, lipases, glucosidases and nucleases with the potential they hydrolyse almost all components of the phototroph's photosynthate. Secreted lipase and peptidase were upregulated in and the presence of *Synechococcus* with fold change of 46.4 and 35.3, respectively (table 4.2.1.1.2), indicating that the DOM produced by *Synechococcus* induces a change in the exoproteome of this heterotroph. Previous analysis reveals that *A. marinum* do not degrade starch, cellulose, chitin and casein and this was detected here as well (Bruns et al., 2003).

Membrane transporters play an important role to get available nutrients from the environment. In *A. marinum*, more membrane transporters were detected in mono-culture (16.71%) as compared to co-culture (9.85%; figure 4.2.1.1.2), possibly caused by a switch into a scavenging lifestyle as the phototroph would provide a constant source of carbon and energy as opposed to the one-off supply of pyruvate in the mono-culture.

As discussed previously, the presence of the cytoplasmic proteins is an indication of cell lysis. In *A. marinum*, a large proportion of these proteins were detected in both conditions (~56% of the exoproteome) indicating that this organism is highly 'leaky', that the growth conditions were not optimal for this organism, or that cells were damaged during sample processing.

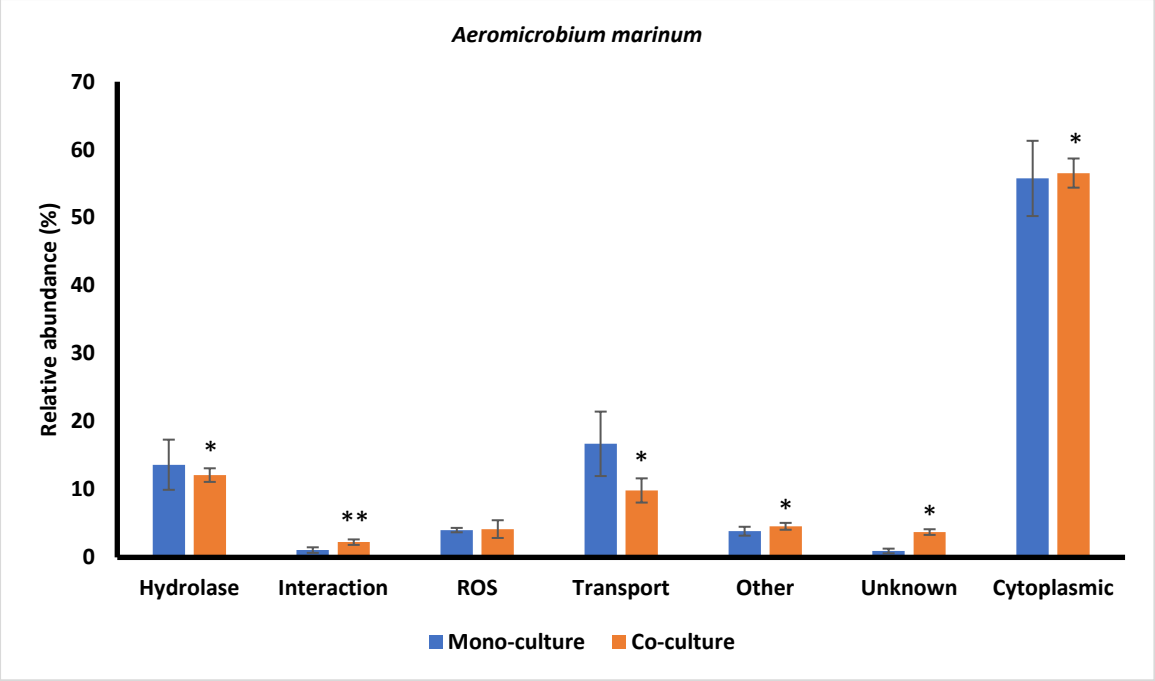

Figure 4.2.1.1.2: Functional category abundance of proteins found in the exoproteome of *A. marinum* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.1.2: List of up and down-regulated proteins of *A. marinum* of co-culture vs mono-culture.

Accession no.	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
WP_007077381.1	Uncharacterized protein	Unknown	81.8	0	0.07	Signal peptide
WP_007077576.1	Secretory lipase	Hydrolase	46.4	0	0.02	Signal peptide
WP_007079377.1	Uncharacterized protein	Unknown	37.8	0	0.02	Non-classical secretion
WP_007077114.1	Peptidase, M23 family	Hydrolase	35.3	0	0.39	Signal peptide
WP_007077252.1	Uncharacterized protein (PQQ-dependent catabolism-associated beta-propeller protein)	Other	25.1	0	0.08	Signal peptide
WP_007077348.1	Hydrolase, NUDIX family	Cytoplasmic	-9.5	0	0.03	>0.1
WP_007079354.1	Uncharacterized protein	Other	-9.8	0	0.00	Signal peptide
WP_007079554.1	Uncharacterized protein	Other	-11.1	0.03	0.03	Signal peptide
WP_007078217.1	50S ribosomal protein L32	Cytoplasmic	-12.1	0.02	0.04	Signal peptide
WP_007078812.1	LPXTG-motif cell wall anchor domain protein	Unknown	-18.8	0	0.01	Signal peptide

4.2.1.2.CFB group: We have included four representatives of this group in this study as they play a key role in the degradation of polymers and, potentially, more recalcitrant DOM.

4.2.1.2.1 *Polaribacter sp*. MED 152: The total number of proteins detected in the exoproteome of *Polaribacter sp*. MED 152 were 427. In the exoproteome of *Polaribacter*, proteases, glucosidases, lipases and phosphatases were detected proving that proteins and complex carbohydrates are an important source of carbon and nitrogen for *Polaribacter* as previously suggested (Gonzalez et al., 2008). Nevertheless, the fact that co-culture with *Synechococcus* did not induce an up-regulation of hydrolytic enzymes (table 4.2.1.2.1 and figure 4.2.1.2.1) suggests this strain may not be adapted to respond to the labile DOM produced by this phototrophic organism. The data even reveals that a sialidase-like enzyme was downregulated in co-culture (-11x fold; table 4.2.1.2.1). Genome analysis of *Polaribacter* reveals that it contains very low number of transporters which makes it a specialized bacterium (Gonzalez et al., 2008); nevertheless, the high abundance of transporters found in the exoproteome (i.e. 27% of active membrane transporters in mono-culture and 22% in co-culture; Figure 4.2.1.2.1) indicates a scavenging lifestyle of this organism, mainly using the TonB dependent and starch binding outer membrane proteins (SusD). TonB dependent transporters are the bacterial outer membrane proteins that bind and transport the substrates by using the energy in the form of proton motive force (Nicholas et al., 2010). As observed in *A. marinum*, cytoplasmic proteins were abundantly detected.

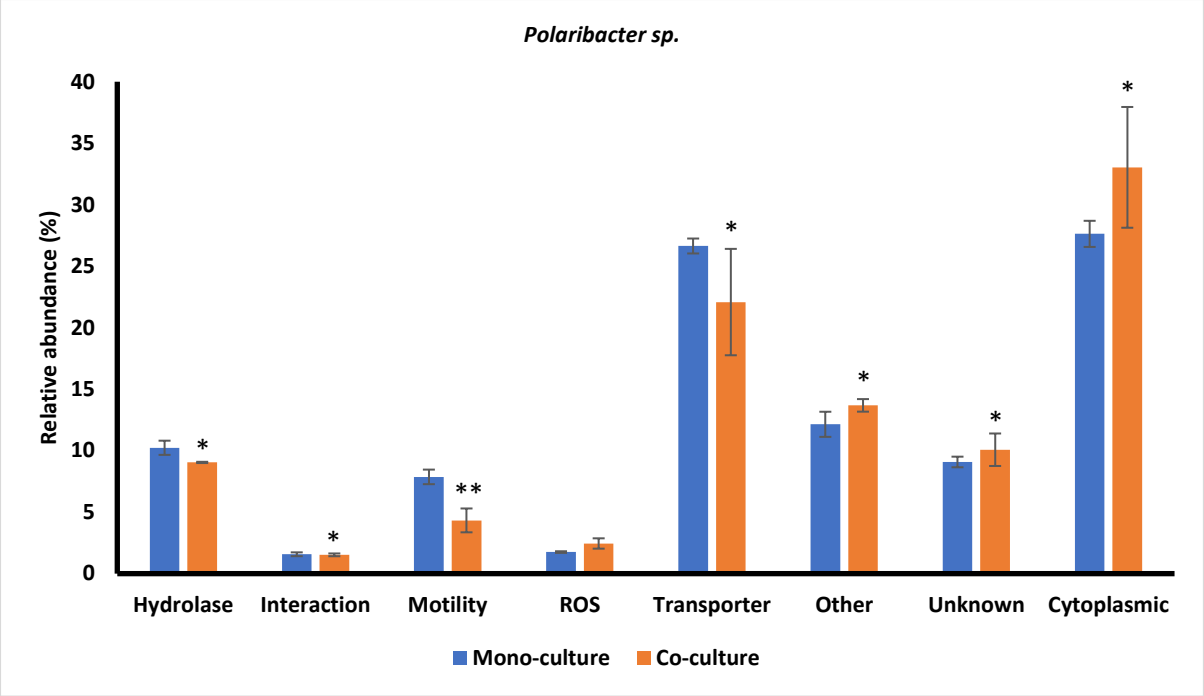

Figure 4.2.1.2.1: Functional category abundance of proteins found in the exoproteome of *Polaribacter* sp. in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.2.1: List of up and down-regulated proteins of *Polaribacter* sp. of co-culture vs mono-culture.

Accession no.	Description	General Category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_007672274.1	Uncharacterized protein	Unknown	16.8	0	0.03	Signal peptide
YP_007670368.1	Chaperone protein DnaJ	Cytoplasmic	7.5	0.01	0.00	Non-classical secretion
YP_007670134.1	Uncharacterized protein (polycystic kidney disease I (PKD) domain)	Unknown	4.7	0	0.07	Non-classical secretion
YP_007670644.1	Peptide methionine sulfoxide reductase MsrA	Other	4.6	0	0.02	Signal peptide
YP_007670631.1	Fumarylacetoacetase	Cytoplasmic	4.2	0	0.34	>0.1
YP_007671584.1	Uncharacterized protein (gliding motility-associated C-terminal domain)	Motility	-3.1	0.01	0.01	Signal peptide
YP_007672082.1	Uncharacterized protein (Periplasmic ligand-binding sensor domain)	Other	-3.3	0	0.02	Non-classical secretion
YP_007672583.1	Uncharacterized protein	Unknown	-4.0	0	0.06	Signal peptide
YP_007670116.1	Uncharacterized protein (carbohydrate-binding modules from Bacteroides thetaiotaomicron SusE, SusF and similar proteins)	Transporter (PUL)	-4.3	0.12	0.01	Signal peptide
YP_007671622.1	Uncharacterized protein (sialidases/neuraminidases)	Hydrolase	-11.0	0	0.01	Signal peptide

4.2.1.2.2 *Algoriphagus machipongonensis* PR1: The total number of proteins detected in *A. machipongonensis* were 321. The hydrolases detected in the exoproteome of *A. machipongonensis* were proteases, glucosidases, phosphatases, sulfatases and amidases. Interestingly, as the peptidase WP_008202063.1 was upregulated in co-culture (12x fold increase; table 4.2.1.2.2), another protease, annotated as a carboxypeptidase-related protein WP_008201653.1, was downregulated (9x fold change). It has been detected previously in *A. machipongonensis* that it has a high capacity to degrade the polysaccharides (Alegado et al., 2010), although *Synechococcus* has been reported to produce more proteins than carbohydrates in its photosynthate (Christie-Oleza et al., 2017).

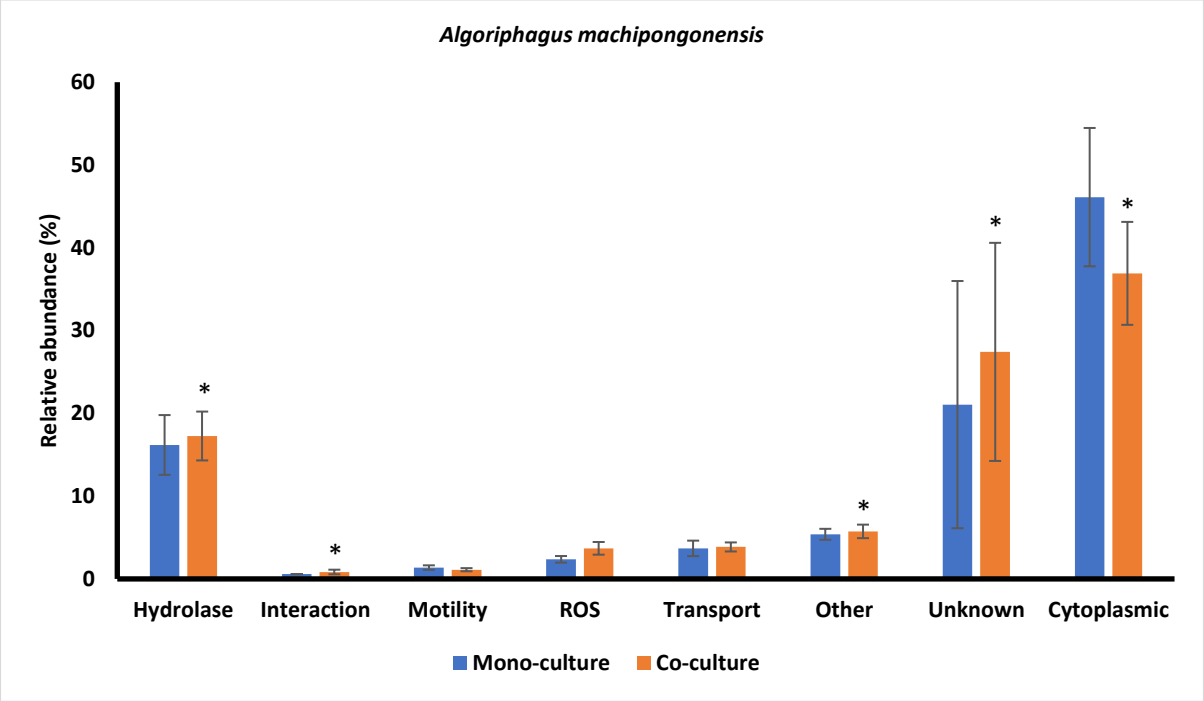

Figure 4.2.1.2.2: Functional category abundance of proteins found in the exoproteome of *A. machipongonensis* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.2.2: List of up and down-regulated proteins of *A. machipongonensis* of co-culture vs mono-culture.

Accession no.	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
WP_008200771.1	O-acetylhomoserine (Thiol)-lyase	Cytoplasmic	28.6	0.04	1.42	>0.1
WP_008200827.1	Uncharacterized protein	Unknown	15.3	0	1.38	Signal peptide
WP_008202063.1	Peptidase, M28 family	Hydrolase	12.2	0.02	0.04	Signal peptide
WP_008197534.1	Fasciclin domain protein	Interaction	11.4	0.03	0.15	Non-classical secretion
WP_008200966.1	Hypothetical cytosolic protein	Unknown	9.9	0.05	0.27	>0.1
WP_008198488.1	50S ribosomal protein L19	Cytoplasmic	-4.7	0.05	0.23	>0.1
WP_008203010.1	50S ribosomal protein L6	Cytoplasmic	-6.8	0.10	0.03	Non-classical secretion
WP_008201653.1	Carboxypeptidase-related protein	Hydrolase	-8.8	0.03	0.02	Signal peptide
WP_008199466.1	Aldehyde dehydrogenase	Cytoplasmic	-14.1	0.02	0.03	>0.1
WP_008198775.1	Ketol-acid reductoisomerase	Cytoplasmic	-38.0	0.02	0.00	Non-classical secretion

4.2.1.2.3 *Gramella forsetii* KT0803: The total number of proteins detected in *G. forsetii* was 142. Interestingly, *G. forsetii* showed an extremely high abundance of proteins involved in hydrolytic activities (i.e. 44% in mono-culture and 36% in co-culture; Figure 4.2.1.2.3). *G. forsetii* is known to contain one of the highest number of glycoside hydrolases per megabase (Bauer et al., 2006). In *G. forsetii*, one of the hydrolases, initially annotated as a hypothetical protein (lipase) but with a role in lipid biodegradation, was strongly upregulated in co-culture (45.4x fold increase; table 4.2.1.2.3). Bacteroidetes species have a large array of CAZymes involved in the polysaccharide utilization (Grondin et al., 2017; Helbert, 2017). Polysaccharides are usually uptaken via PUL transport systems which are encoded in defined operons. In *G. forsetii*, PUL transporters are activated by laminarin and alginate (Kabisch et al., 2014) and other Flavobacteria increase the abundance of their SusD and TonB dependent transporters during phytoplankton blooms (Teeling et al., 2012, 2016). Our data show that these transporters are also induced by the presence of *Synechococcus* photosynthate (i.e. the relative abundance of transporters increased from 5% in mono-culture to almost 21% in co-culture; figure 4.2.1.2.3), being mostly TonB dependent and SusD-like transporters. One

of these SusD transporters was highly upregulated in co-culture with a 17.3 x fold increase (table 4.2.1.2.3).

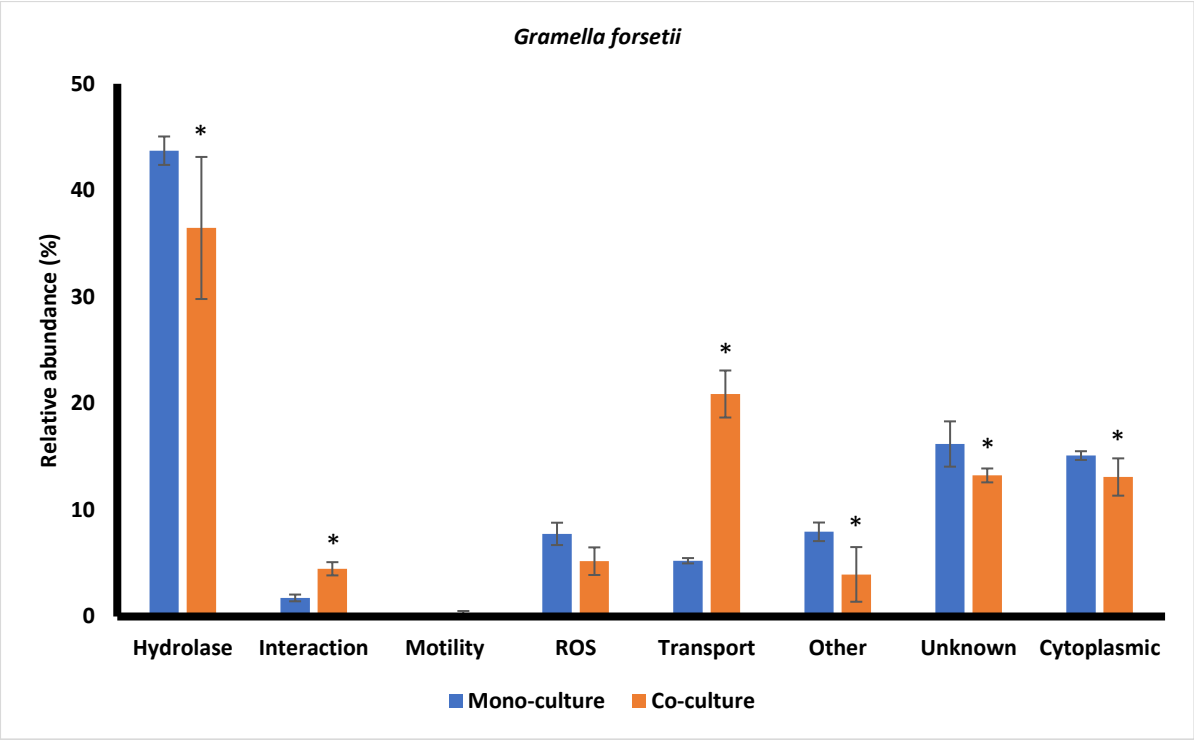

Figure 4.2.1.2.3: Functional category abundance of proteins found in the exoproteome of *G. forsetii* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.2.3: List of up and down-regulated proteins of *G. forsetii* of co-culture vs mono-culture.

Accession no.	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_862367.1	fasciclin domain-containing protein	Interaction	207.2	0	2.48	Signal peptide
YP_860954.1	hypothetical protein GFO_0913	Unknown	49.9	0	0.57	Signal peptide
YP_860281.1	hypothetical protein GFO_0220 (lipases and esterases)	Hydrolase	45.4	0	0.70	Non-classical secretion
YP_862935.1	SusD/RagB family protein	Transport (PUL)	17.3	0	11.04	>0.1
YP_862095.1	hypothetical protein GFO_2063	Unknown	15.3	0	1.29	Non-classical secretion
YP_860804.1	zinc-type alcohol dehydrogenase	Other	-7.8	0.07	0.55	>0.1
YP_863381.1	thrombospondin repeat-containing OmpA/MotB family outer membrane protein	Other	-7.9	0	0.06	Signal peptide
YP_861611.1	hypothetical protein GFO_1571 (Dodecin ;Dodecin is a flavin-binding protein).	Other	-10.8	0.10	0.30	>0.1
YP_862184.1	carboxylesterase	Hydrolase	-12.6	0.07	0.16	Signal peptide
YP_862162.1	glyceraldehyde-3-phosphate dehydrogenase	Cytoplasmic	-25.9	0	0.05	Non-classical secretion

4.2.1.2.4 *Formosa agariphila* KMM3901: The total number of proteins detected in *F. agariphila* was 311. This strain also produced a high relative abundance of hydrolytic enzymes (27% and 22% in mono-culture and co-culture, respectively; figure 4.2.1.2.4). As previously reported, *F. agariphila* has an alga-associated lifestyle because of its ability to degrade a wide range of algal polysaccharides (Mann et al., 2013). Here, no hydrolases were strongly upregulated in the presence of *Synechococcus* and even an alginate lyase was strongly down-regulated by 20x fold (table 4.2.1.2.4), again supporting the idea that this marine cyanobacterium does not produce large amounts of polysaccharides and, hence, does not support strong interactions with the CFB taxonomic group.

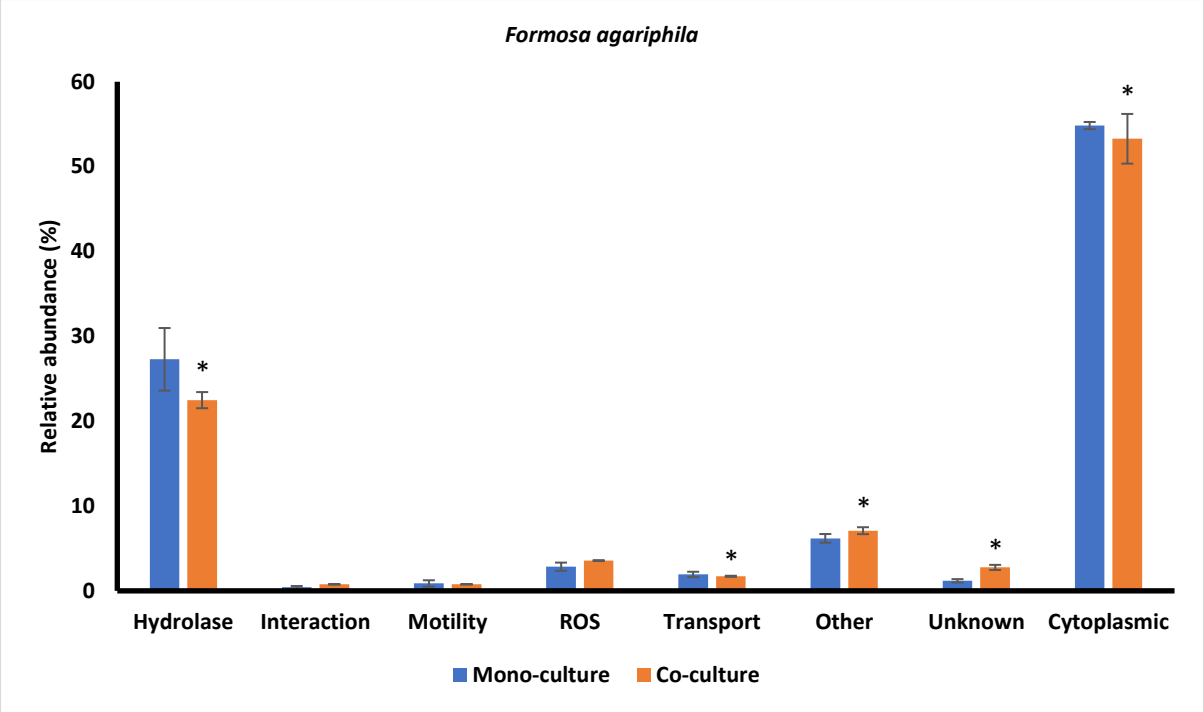

Figure 4.2.1.2.4: Functional category abundance of proteins found in the exoproteome of *F. agariphila* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.2.4: List of up and down-regulated proteins of *F. agariphila* of co-culture vs mono-culture.

Accession no.	Description	General Category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
CDF79125	alkyl hydroperoxide reductase	ROS	46.4	0.05	0.19	Non-classical secretion
CDF78285	50S ribosomal protein L24	Cytoplasmic	12.7	0.12	0.21	Non-classical secretion
CDF80120	50S ribosomal protein L20	Cytoplasmic	12.5	0.05	0.03	Non-classical secretion
CDF78237	conserved hypothetical protein (DUF1573) containing PapD-like domain, putative periplasmic chaperone protein	Unknown	11.5	0.05	0.10	Signal peptide
CDF80405	conserved hypothetical protein (DUF500) containi ng Ysc84 actin-binding domain (The SYLF domain (also called DUF500), a novel lipid-binding module)	Transport	10.8	0.04	0.03	Signal peptide
CDF80355	aldehyde dehydrogenase	Cytoplasmic	-8.6	0	0.12	>0.1
CDF79988	PKD domain-containing protein, partial	Other	-9.5	0.02	0.01	Non-classical secretion
CDF80366	alginate lyase (PL6/PL7/CBM32)	Hydrolase	-20.5	0.01	0.00	Signal peptide
CDF79251	conserved hypothetical protein (Sortilin, neurotensin receptor 3)	Other	-22.7	0	0.01	Signal peptide
CDF78751	chondroitin AC/alginate lyase family protein (PL 8), partial	Hydrolase	-26.5	0	0.01	Non-classical secretion

4.2.1.3 Alphaproteobacteria: It has been previously reported that Roseobacters produces just a small number of largely uncharacterised hydrolytic enzymes to degrade the dissolved organic matter which is produced by phototrophs (Christie-Oleza et al., 2015). The extracellular hydrolytic potential is, hence, potentially very low in marine alphaproteobacteria due to low number of enzymes in their genome (Barbeyron et al., 2016) although it was proven to be highly efficient in recycling nutrients from photosynthate (Christie-Oleza et al., 2017). Previous analysis of Roseobacter strain's exoproteome revealed that different isolates produced a small but highly divergent repertoire of exoenzymes, suggesting that closely related species may be able to target different substrates and escape competition through the diversification of resources (Christie-Oleza et al., 2015b).

4.2.1.3.1 *Ruegeria pomeroyi* DSS-3: The number of proteins detected in *R. pomeroyi* was 371. As expected, the abundance of the hydrolytic enzymes in the exoproteome of *R.*

pomeroyi was low (i.e. 2.3% in mono-culture with a small increase to 4% in co-culture; figure 4.2.1.3.1). These hydrolytic enzymes are presumably involved in degrading polysaccharides, proteins and lipids, although resolving their poor characterisation is the purpose of chapter 5 of this thesis. The alkaline phosphatase PhoX was abundantly detected in the exoproteome of *R. pomeroyi* (i.e. 0.19% in mono-culture with an increase to 0.56% in co-culture) suggesting a key role of this enzyme in regenerating organic phosphorous into phosphate. The substrates that are targeted by PhoX were previously characterized (Sebastian and Ammerman, 2011).

The motility of *R. pomeroyi* was discussed in Chapter 3. Here, in this experiment, the abundance of motility related proteins in the exoproteome of *R. pomeroyi* was 5.6% in mono-culture and 7.5% in co-culture, suggesting an increase in motility in the presence of small amounts of DOM but, due to the short incubation period (i.e. 3 days) and, unlike in chapter 3, it is difficult to extract conclusions from the information on just a single time point. Roseobacters are known to scavenge a large variety of scarce nutrients in oligotrophic marine systems and to proliferate rapidly in nutrient-rich patches of the ocean (Moran et al., 2004; Newton et al., 2010; Christie-Oleza et al., 2012a, b) and, hence, active membrane transporters are usually abundantly detected in the exoproteome of *R. pomeroyi* (Christie-Oleza et al., 2015). Here, over 20% in mono-culture and 33% in co-culture was made of these proteins (figure 4.2.1.3.1). Interestingly, three ABC transporters were highly up-regulated in the presence of *Synechococcus* (table 4.2.1.3.1), mainly for amino acids, highlighting *R. pomeroyi*'s preference for nitrogen-containing organic compounds (table 4.2.1.3.1).

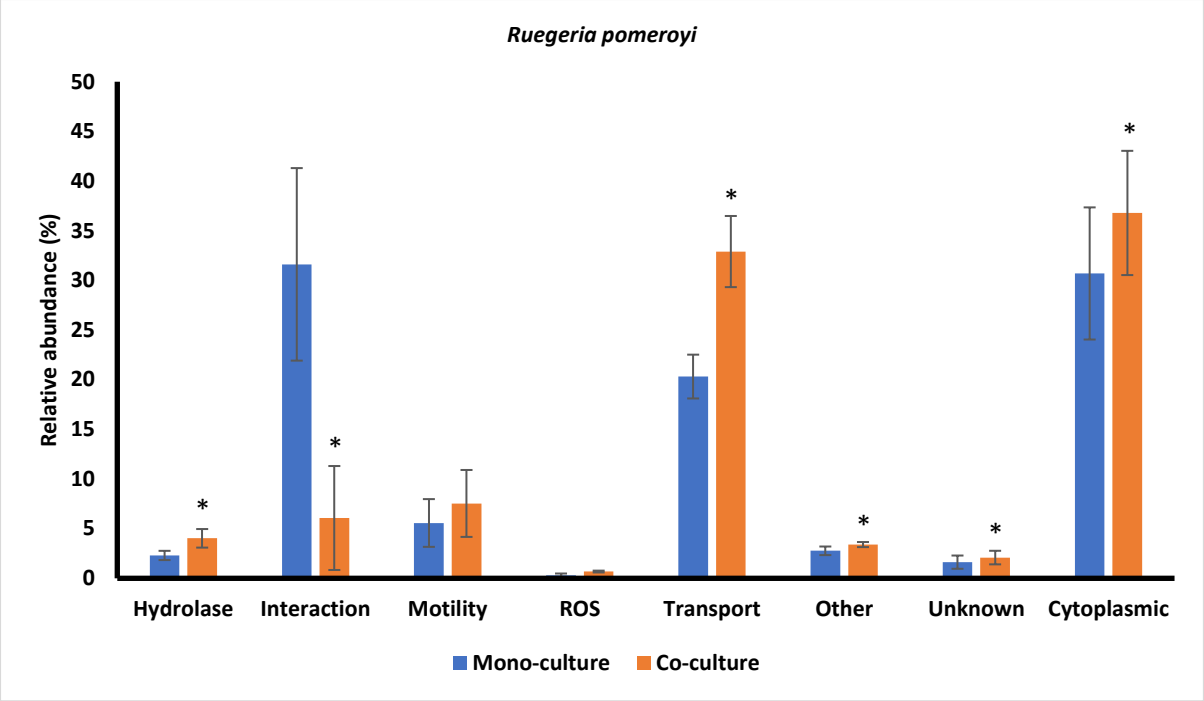

Figure 4.2.1.3.1: Functional category abundance of proteins found in the exoproteome of *R. pomeroyi* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.3.1: List of up and down-regulated proteins of *R. pomeroyi* of co-culture vs mono-culture.

Accession no.	Description	General Category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
AAV96342	hypothetical protein SPO3107 (NAD(P)+-dependent aldehyde dehydrogenase superfamily)	Other	215.9	0	0.35	Signal peptide
AAV94993	branched-chain amino acid ABC transporter, periplasmic substrate-binding protein	Transport	46.8	0	0.08	Signal peptide
AAV94835	glycine betaine/proline ABC transporter, periplasmic substrate-binding protein	Transport	45.2	0	0.05	Signal peptide
AAV95695	glycine betaine/proline ABC transporter, periplasmic glycine betaine/proline-binding protein	Transport	45.2	0	0.04	Signal peptide
AAV96343	peptidoglycan-associated lipoprotein	Other	45.1	0	0.07	Signal peptide
AAV94458	hypothetical protein SPO1159	Unknown	-8.1	0.01	0.02	Signal peptide
AAV93552	PaxA, putative	Interaction	-8.7	0.04	4.88	Non-classical secretion
AAV96581	phosphoserine aminotransferase	Cytoplasmic	-10.2	0	0.06	>0.1
AHB86024	Hypothetical protein	Unknown	-24.0	0	0.01	Signal peptide
AAV93822	ribosomal protein L15	Cytoplasmic	-25.3	0.02	0.01	Non-classical secretion

4.2.1.3.2 *Roseobacter denitrificans* och114: The number of proteins detected in the exoproteome of *R. denitrificans* was 276. Proteins involved in interaction were abundant in *R. denitrificans* (~10%; figure 4.2.1.3.2) but not differentially detected between mono- and co-cultures. This category includes GTA and RTX-like proteins which were discussed in chapter 3. GTA are bacteriophage like particles which are responsible of horizontal gene transfer (Frost et al., 2005) and are commonly found in Roseobacter isolates (Biers et al., 2008; Zhao et al., 2009). RTX-like elements play an important role in cell-cell interactions by either adhesion or toxicity, but the function is still largely unknown (Linhartova et al., 2010).

The abundance of motility related proteins, mainly flagelin, was over 18% in mono-culture and 15% in co-culture. Again, transporters were abundantly detected but, interestingly, they were much more abundant in co-culture (43%) than in mono-culture (15%) which may suggest this strain responds to the large diversity of substrates within the DOM produced by *Synechococcus*. (figure 4.2.1.3.2). Two transporters are particularly up-regulated in co-culture (table 4.2.1.3.2), particularly for an undefined carbohydrate and putrescine (45.7x and 30.9x fold increase, respectively). Putrescine is a compound that is highly abundant in most marine phototrophs.

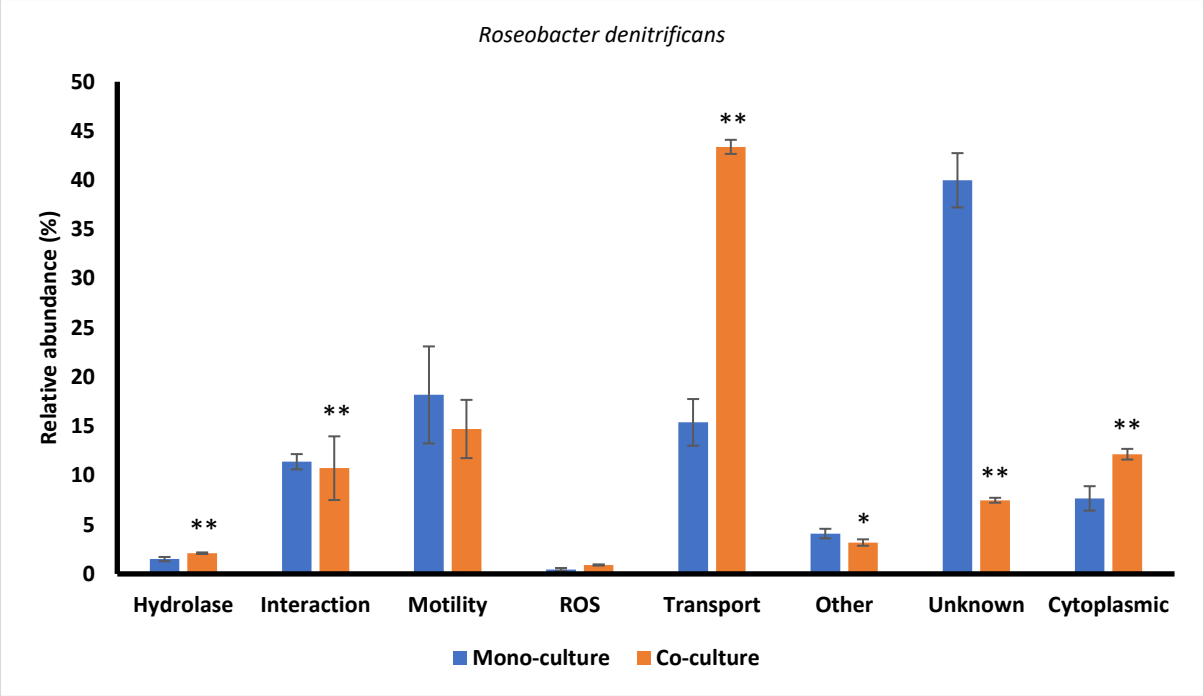

Figure 4.2.1.3.2: Functional category abundance of proteins found in the exoproteome of *R. denitrificans* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.3.2: List of up and down-regulated proteins of *R. denitrificans* of co-culture vs mono-culture.

Accession no.	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_680873.1	hypothetical protein RD1_0477	Unknown	142.5	0	0.42	Non-classical secretion
YP_683229.1	phage major capsid protein	Interaction	71.0	0	0.30	Non-classical secretion
YP_681400.1	C4-dicarboxylate transport system, substrate-binding protein	Transport	45.7	0	0.30	Signal peptide
YP_681726.1	50S ribosomal protein L4	Cytoplasmic	35.4	0	0.46	>0.1
YP_680588.1	putrescine ABC transporter substrate-binding protein	Transport	30.9	0	0.29	Signal peptide
YP_681001.1	hypothetical protein RD1_0615 (VPLPA-CTERM protein sorting domain)	Interaction	-26.0	0	0.04	Signal peptide
YP_681739.1	hypothetical protein RD1_1414 (VPLPA-CTERM protein sorting domain)	Interaction	-26.4	0	0.23	Signal peptide
YP_684134.1	iron-regulated protein frpC	Interaction	-35.5	0	0.00	Non-classical secretion
YP_682808.1	hypothetical protein RD1_2565	Unknown	-116.2	0	0.13	Signal peptide
YP_682797.1	hypothetical protein RD1_2554	Unknown	-132.0	0	0.03	Non-classical secretion

4.2.1.3.3 *Dinoroseobacter shibae* DFL-12: The total proteins detected in *D. shibae* were 355. In *D. shibae*, the abundance of proteins involved in motility was very high, with a strong increase from mono-culture (38%) to co-culture (63%). Motility plays an important role in the scavenging of Roseobacters for DOM (chapter 3 and Bartling et al., 2018) and phytoplankton interactions. *D. shibae* was isolated from its association with a dinoflagellate and is known for swimming and attaching to eukaryotic phytoplankton (Wang et al., 2014). Hence, they switch from a motile to a sessile lifestyle by the loss of flagella and the formation of biofilms (Geng and Belas, 2010). In *D. shibae*, the abundance of transporters was lower than that observed in the other two Roseobacter strains used in this study (12.6% in mono-culture and 15.5% in co-culture; figure 4.2.1.3.3). Interestingly, three transporters were upregulated in co-culture for which the specific substrate is unknown and deserves further investigation (table 4.2.1.3.3).

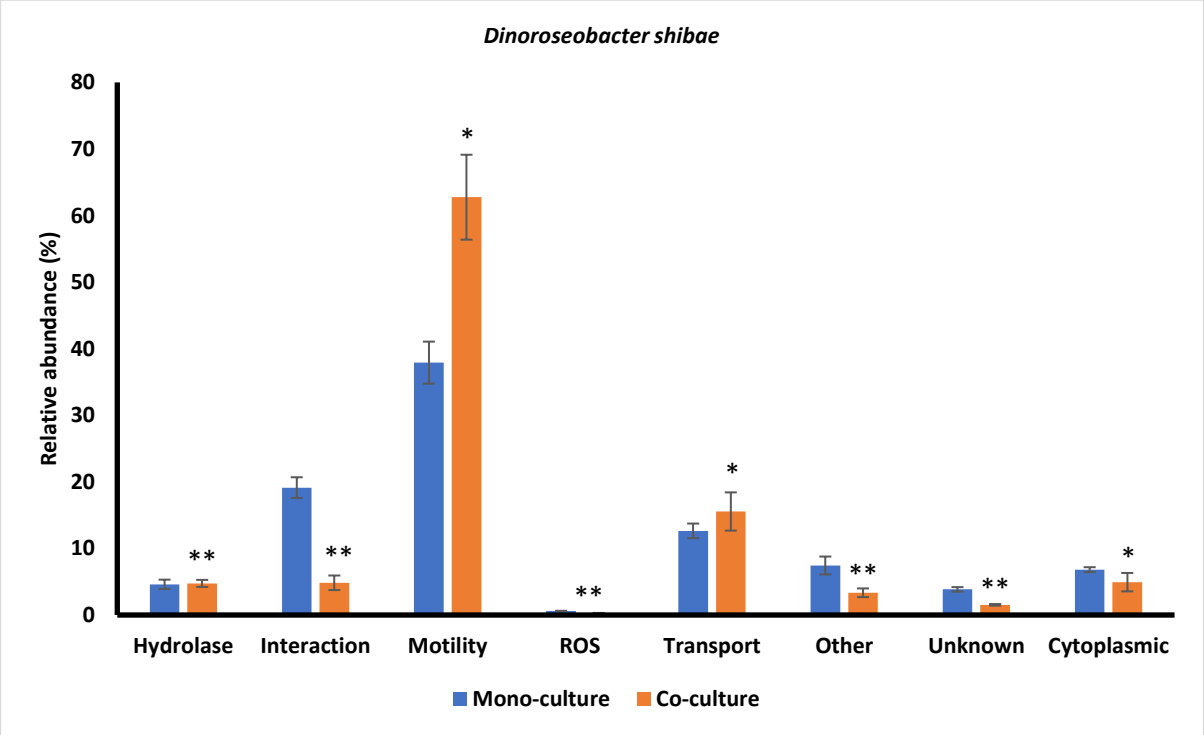

Figure 4.2.1.3.3: Functional category abundance of proteins found in the exoproteome of *D. shibae* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.3.3: List of up and down-regulated proteins of *D. shibae* of co-culture vs mono-culture.

Accession no	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_001533006.1	periplasmic-binding protein-like II	Transport	15.2	0	0.04	Signal peptide
YP_001542003.1	extracellular solute-binding protein	Transport	9.1	0	0.07	Signal peptide
YP_001533913.1	hypothetical protein Dshi_2579	Unknown	8.2	0	0.20	Signal peptide
YP_001533680.1	hypothetical protein Dshi_2343	Transport	6.1	0.01	2.79	Signal peptide
YP_001532740.1	phospholipase/lecithinase/hemolysin-like protein	Hydrolase	5.9	0	0.32	>0.1
YP_001533224.1	hypothetical protein Dshi_1881	Unknown	-64.2	0	0.00	Signal peptide
YP_001532246.1	hypothetical protein Dshi_0900 (it's involved in a wide variety of important cellular functions)	Interaction	-69.0	0	0.00	Signal peptide
YP_001534114.1	hypothetical protein Dshi_2780	Unknown	-80.2	0	0.00	Signal peptide
YP_001531917.1	cobalamin/Fe3+-siderophores ABC transporter protein	Transport	-104.1	0	0.00	Non-classical secretion
YP_001542145.1	ErfK/YbiS/YcfS/YnhG family protein (Lipoprotein-anchoring transpeptidase)	Other	-134.2	0	0.00	Signal peptide

4.2.1.4 Gammaproteobacteria

4.2.1.4.1 *Pseudoalteromonas citrea* NCMB1889: The total number of proteins that were detected in *P. citrea* was 217. As expected from a generalist Gammaproteobacteria, a large abundance of hydrolytic enzymes were detected (i.e. 43.7% in mono-culture which increased to 61.1% in co-culture; figure 4.2.1.4.1). An alpha amylase, alkaline phosphatase and collagenase was up-regulated in co-culture as shown in table 4.2.1.4.1. As observed in other heterotrophic organisms, an alpha amylase was enormously upregulated in the presence of *Synechococcus* (999x fold increase), a finding that requires further investigation to confirm the substrate of these, theoretically, starch-degrading enzymes.

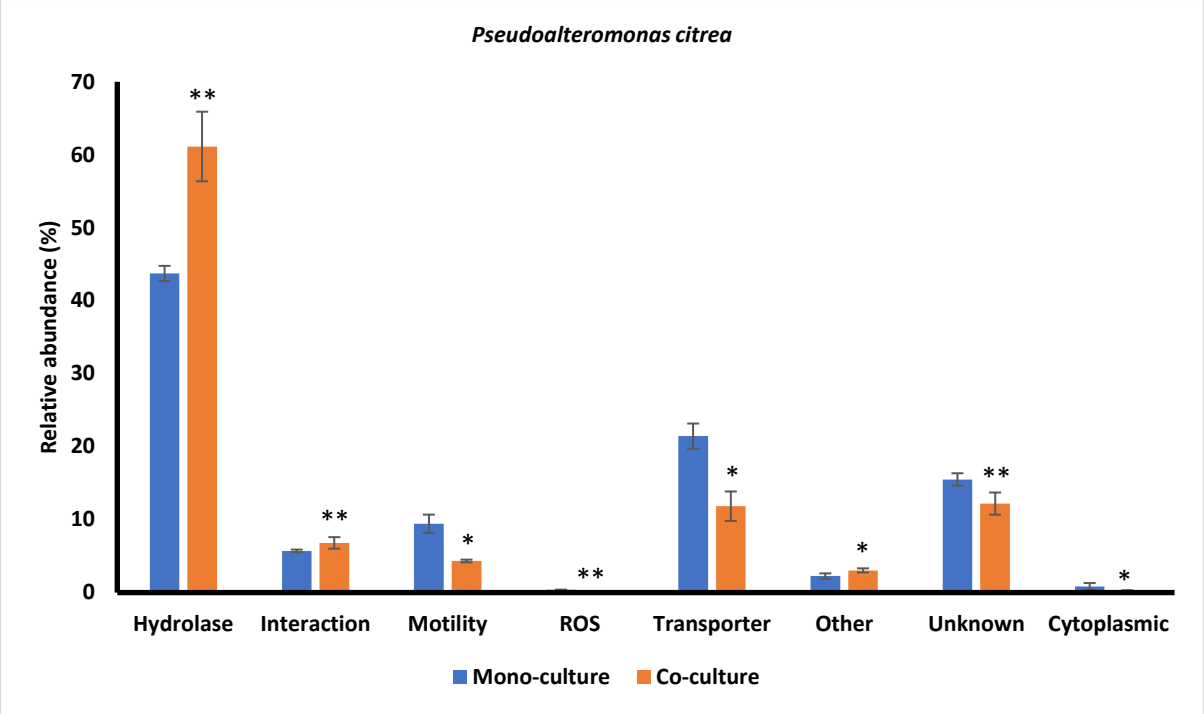

Figure 4.2.1.4.1: Functional category abundance of proteins found in the exoproteome of *P. citrea* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.4.1: List of up and down-regulated proteins of *P. citrea* of co-culture vs mono-culture.

Accession no	Description	General Category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
WP_010365747.1	alpha-amylase	Hydrolase	998.6	0	0.89	Signal peptide
WP_010361928.1	alkaline phosphatase	Hydrolase	57.2	0	0.03	Signal peptide
WP_010367581.1	collagenase	Hydrolase	53.0	0	0.03	Signal peptide
WP_010362685.1	collagenase	Hydrolase	43.2	0	0.03	Signal peptide
WP_010364195.1	porin	Other	37.5	0	0.20	Signal peptide
WP_010364122.1	peptidase	Hydrolase	-111.0	0	0.00	Signal peptide
WP_010361520.1	hypothetical protein	Unknown	-117.8	0	0.00	Signal peptide
WP_010362945.1	hypothetical protein	ROS	-147.0	0	0.00	Signal peptide
WP_010362105.1	bacterioferritin	Transporter	-206.7	0	0.01	>0.1
WP_010367046.1	branched-chain amino acid aminotransferase	Cytoplasmic	-367.5	0	0.00	>0.1

4.2.1.4.2 *Alteromonas macleodii* ATCC27126: The total number of proteins that were detected in *A. macleodii* was 132. The abundance of hydrolytic enzymes in the exoproteome of *A. macleodii* was not as high as in *P. citrea* and, in this case, the presence of the phototroph decreased the relative abundance of hydrolases produced by this strain (24.6% in mono-culture *versus* 9.4% in co-culture, figure 4.2.1.4.2). It has already been reported in *A. macleodii* that it encodes a considerable amount of CAZymes to degrade polymers of phytoplankton exudates and allows its growth on these polymeric substrates (Neumann et al., 2015). Despite the overall decrease in abundance of hydrolases, a xylanase was up-regulated in *A. macleodii* in the presence of the cyanobacterium with a 25.7x fold increase (table 4.2.1.4.2). Again, it is uncertain whether *Synechococcus* produces a xylan-like polysaccharide or if this enzyme may hydrolase other polysaccharides produced by the phototroph. *A. macleodii* is a motile organism because of the presence of one unsheathed polar flagellum (Gonzaga et al., 2012). There was a very high abundance of motility related proteins in *A. macleodii* (38.4% in mono-culture and 60.9% in co-culture, figure 4.2.1.4.2). The increase in motility in the presence of *Synechococcus* may suggest an induced change in the bacterium's lifestyle.

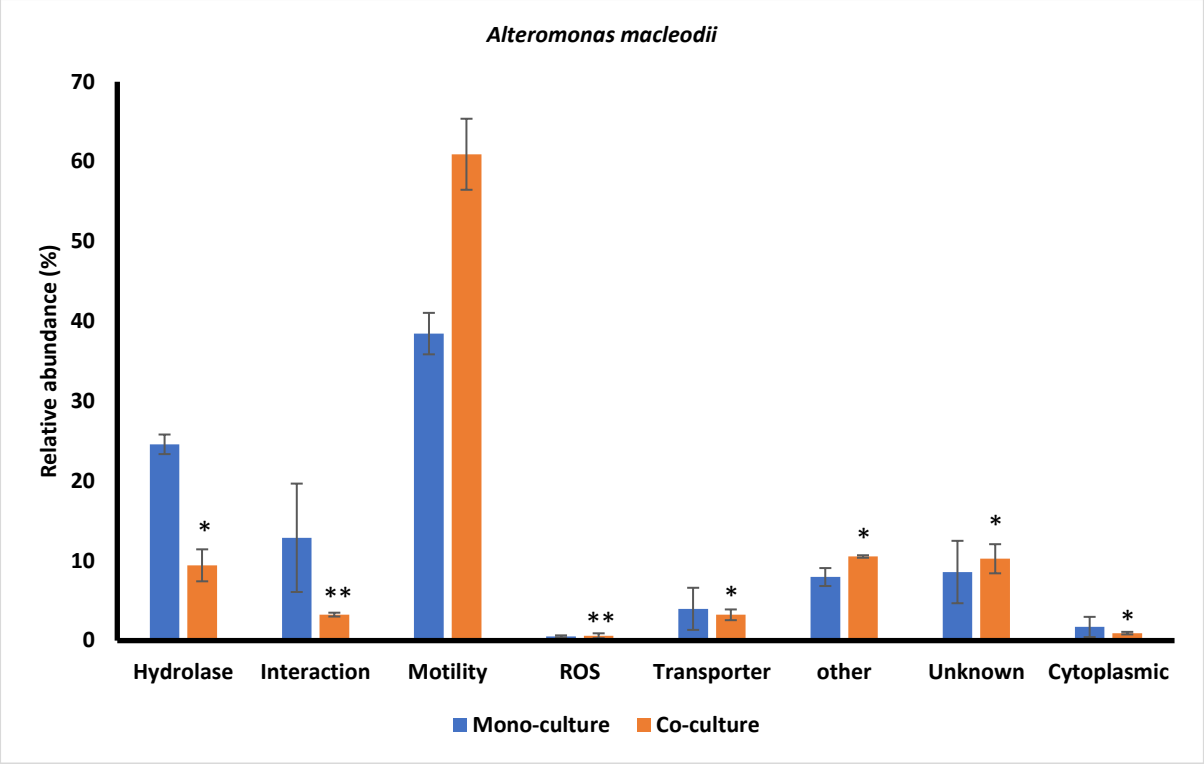

Figure 4.2.1.4.2: Functional category abundance of proteins found in the exoproteome of *A. macleodii* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.4.2: List of up and down-regulated proteins of *A. macleodii* of co-culture vs mono-culture.

Accession no.	Description	General Category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_006746683.1	thiol:disulfide interchange protein DsbA	other	73.3	0.02	0.22	Signal peptide
YP_006748009.1	hypothetical protein MASE_09620 (periplasmic molecular chaperone)	other	45.3	0.01	1.05	Signal peptide
YP_006747774.1	twin-arginine translocation pathway signal protein	other	36.0	0.06	0.44	Non-classical secretion
YP_006749803.1	high-potential iron sulfur protein	other	33.7	0.03	0.99	Non-classical secretion
YP_006749872.1	endo-1,4-beta-xylanase	Hydrolase	25.7	0.03	0.32	Signal peptide
YP_006749394.1	hypothetical protein MASE_16740	Unknown	-29.0	0.03	0.03	Signal peptide
YP_006747913.1	pullulanase	Hydrolase	-36.4	0.02	0.02	Non-classical secretion
YP_006749066.1	glutamine synthetase	Cytoplasmic	-45.1	0	0.02	>0.1
YP_006748129.1	lipoprotein	Unknown	-53.5	0.05	0.01	Signal peptide
YP_006747925.1	penicillin acylase-like protein	other	-97.1	0	0.03	Non-classical secretion

4.2.1.4.3 *Marinobacter adhaerens* HP15: The total number of proteins that were detected in *M. adhaerens* was 186. The average abundance of hydrolases was 6.3% in mono-culture and increased to 13.6% in co-culture (figure 4.2.1.4.3). The interaction between *M. adhaerens* and diatoms has been previously studied showing that this strain plays an important role in the development and aggregation of phytoplankton in marine environments (Gardes et al., 2010). This strain is also used as a model organism to study heterotroph and phytoplankton interactions because they attach to diatoms and help in the formation of marine snow (Gardes et al., 2010, Fowler and Knauer, 1986). Hence, in *M. adhaerens*, adhesion related proteins were detected with abundances between 2.9% in mono-culture and 2.6% in co-culture. It has been suggested previously that *M. adhaerens* can attach to diatom cells and interact with it by using the carbohydrates produced by diatoms as a source of carbon and energy (Gardes et al., 2010). So, the detection of adhesion related proteins is not surprising, although previous analyses in the Christie-Oleza laboratory showed that, while this strain may attach to larger eukaryotic phototrophs, it does not seem to attach to *Synechococcus*. Previous analysis revealed that *M. adhaerens* is motile and contains single polar flagellum and flagella-associated gene clusters were also detected in its genome (Gardes et al., 2010). Here, a high abundance of motility related proteins were detected in the exoproteome of *M. adhaerens* (i.e. 26.99% in mono-culture and increased to 41.24% in co-culture). This may support the imaging observations of no apparent attachment of this heterotroph to small marine cyanobacteria, i.e. *Synechococcus*. The abundance of transporters was also high in the exoproteome of *M. adhaerens* (39.1% in mono-culture and 24.8% in co-culture). Two of *M. adhaerens* transporters were particularly highly up-regulated in co-culture as mentioned in table 4.2.1.4.3. The highly upregulated ABC transporter for urea possibly indicates the build-up of urea in the media which may be a sub-product from purine degradation as *Synechococcus* do not produce urea. It has been previously reported that *M. adhaerens* benefits from the diatom *Thalassiosira weissflogii* by uptaking the amino acids released by the diatom. This was indicated by the up-regulation of transporters involved in amino acid uptake and, interestingly, by the down-regulation of urea transporters (Stahl and Ullrich, 2016). Nevertheless, here the

strong upregulation of the transporter for urea indicates that *M. adhaerens* uses this nitrogen-rich compound as a nitrogen source in co-culture with *Synechococcus*.

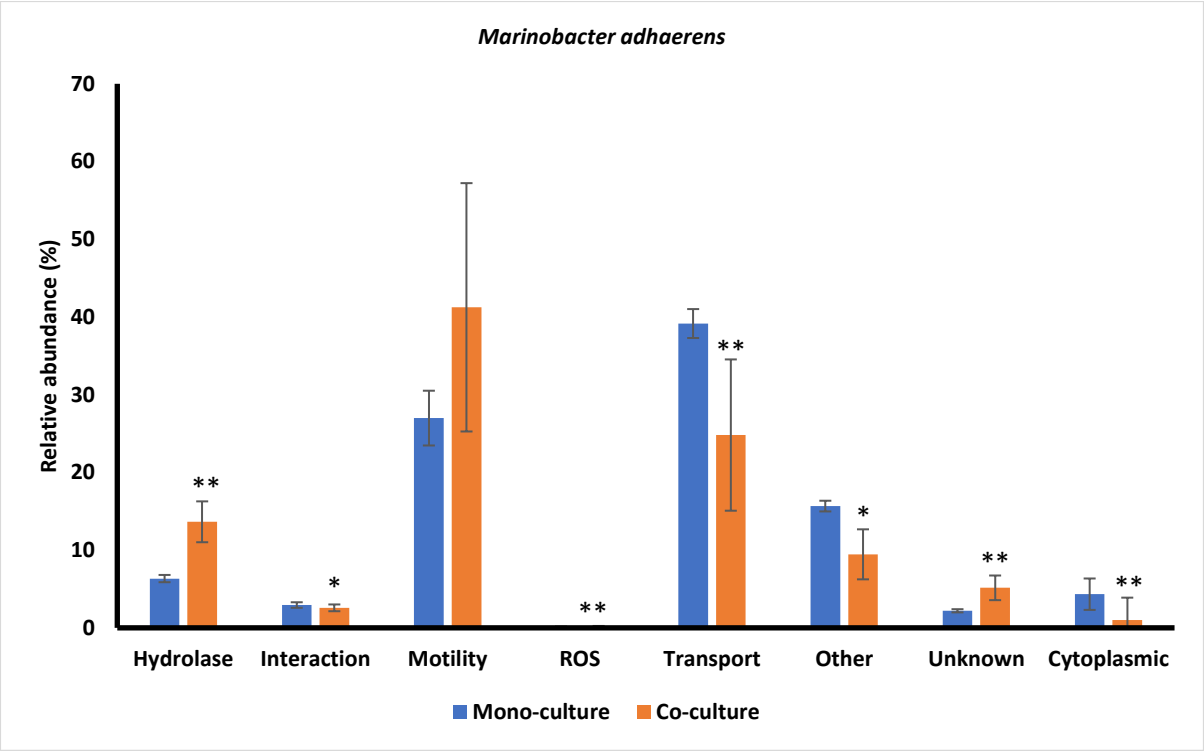

Figure 4.2.1.4.3: Functional category abundance of proteins found in the exoproteome of *M adhaerens* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.4.3: List of up and down-regulated proteins of *M. adhaerens* of co-culture vs mono-culture.

Accession no	Description	General Category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_005887350.1	urea ABC transporter, urea binding protein	Transport	1547.9	0	0.96	Signal peptide
YP_005885541.1	hypothetical protein HP15_1849	Unknown	274.7	0	0.45	Signal peptide
YP_005886842.1	ABC transporter substrate-binding protein	Transport	22.5	0	0.10	Signal peptide
YP_005884520.1	hypothetical protein HP15_828	Unknown	8.8	0	0.33	Non-classical secretion
YP_005887590.1	hypothetical protein HP15_3898	Unknown	6.1	0.01	1.36	Signal peptide
YP_005884192.1	hypothetical protein HP15_500	Unknown	-38.9	0	0.00	Non-classical secretion
YP_005884075.1	50S ribosomal protein L2	Cytoplasmic	-41.5	0	0.00	Non-classical secretion
YP_005886622.1	long-chain fatty acid transport protein	Other	-64.5	0	0.00	Signal peptide
YP_005887437.1	hypothetical protein HP15_3745	Other	-68.1	0	0.00	Signal peptide
YP_005885947.1	glyceraldehyde-3-phosphate dehydrogenase, type I	Cytoplasmic	-85.2	0	0.00	>0.1

4.2.1.4.4 *Pseudomonas stutzeri* AN10: The total number of proteins that were detected in *P. stutzeri* was 266. In the exoproteome of this Gammaproteobacterium the abundance of hydrolases was low (i.e. <1% in both mono- and co-culture; figure 4.2.1.4.4). Nevertheless, an aminopeptidase did show an up-regulation in co-culture (table 4.2.1.4.4) allowing this strain to degrade the proteins produced by *Synechococcus* by removing the amino acid from the N-terminus. This strain is a model organism to study naphthalene and salicylate biodegradation (Rossello´-Mora et al., 1994) although, as expected, no aromatic compounds were expected to be produced by *Synechococcus*. Proteins involved in motility and membrane transport predominated within *P. stutzeri*'s exoproteome. Flagellar proteins were among the most abundant proteins (i.e. 33% in mono-culture and 45.1% in co-culture). It has been previously shown that all species of *Pseudomonas* are motile by having one or more polar flagella (Lalucat et al., 2006). The abundance of membrane transporters was 29.2% in mono-culture and 32.2% in co-culture. Interestingly, four ABC transporters for amino acid were amongst the most highly up-regulated proteins by the presence of *Synechococcus* (table 4.2.1.4.4) suggesting amino acids within the photosynthate are targeted by this Gammaproteobacterium.

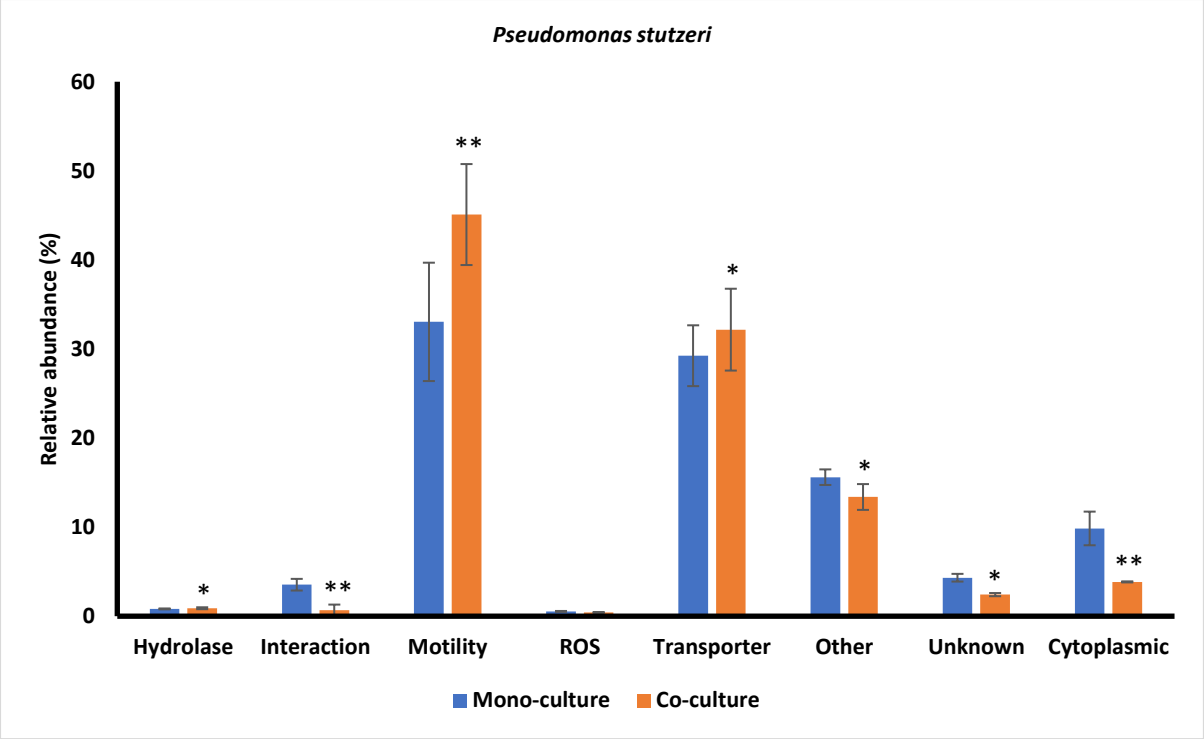

Figure 4.2.1.4.4: Functional category abundance of proteins found in the exoproteome of *P. stutzeri* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.4.4: List of up and down-regulated proteins of *P. stutzeri* of co-culture vs mono-culture.

Accession no.	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_006456254.1	branched-chain amino acid ABC transporter substrate-binding protein	Transporter	358.9	0	0.49	Signal peptide
YP_006457249.1	branched-chain amino acid ABC transporter substrate-binding protein	Transporter	22.6	0	0.04	Non-classical secretion
YP_006457830.1	ABC transporter substrate-binding protein	Transporter	22.4	0	0.09	Signal peptide
YP_006458744.1	aminopeptidase	Hydrolase	19.5	0	0.22	Signal peptide
YP_006455858.1	amino acid ABC transporter periplasmic protein	Transporter	14.1	0	0.04	Signal peptide
YP_006459249.1	30S ribosomal protein S5	Cytoplasmic	-163.0	0	0.00	Non-classical secretion
YP_006459265.1	50S ribosomal protein L4	Cytoplasmic	-171.5	0	0.00	Non-classical secretion
YP_006459266.1	50S ribosomal protein L3	Cytoplasmic	-180.1	0	0.00	Non-classical secretion
YP_006459252.1	30S ribosomal protein S8	Cytoplasmic	-231.0	0	0.00	Non-classical secretion
YP_006459267.1	30S ribosomal protein S10	Cytoplasmic	-393.9	0	0.00	>0.1

4.2.1.5 Planctomycetes: This group is well known for its role in global carbon and nitrogen cycles.

4.2.1.5.1 *Planctomyces limnophilus* ATCC43296: The total number of proteins detected in *Planctomyces limnophilus* ATCC43296 was only 30. In *P. limnophilus*, the hydrolases detected were nucleases, lipases and sulfatase. Unexpectedly, their abundance was low (3.3% in mono-culture and 0.8% in co-culture; figure 4.2.1.5.1). The low abundance of secreted hydrolytic enzymes may suggest that *P. limnophilus* does not target polymers as a source of carbon and energy or that is has different mechanisms to uptake these substrates and indicated by the extremely high abundance of proteins of unknown function (>30% of the exoproteome). Planctomyces are largely understudied organisms and the molecular mechanisms behind their involvement in important processes remains unstudied. Proteins involved in interactions were much more abundant in mono-culture than in co-culture (22.4% and 3.7%, respectively). This was mainly driven by phage major capsid protein. Flagellin protein was detected with an average abundance of 5.7% and 8.86% in mono- and co-culture, respectively.

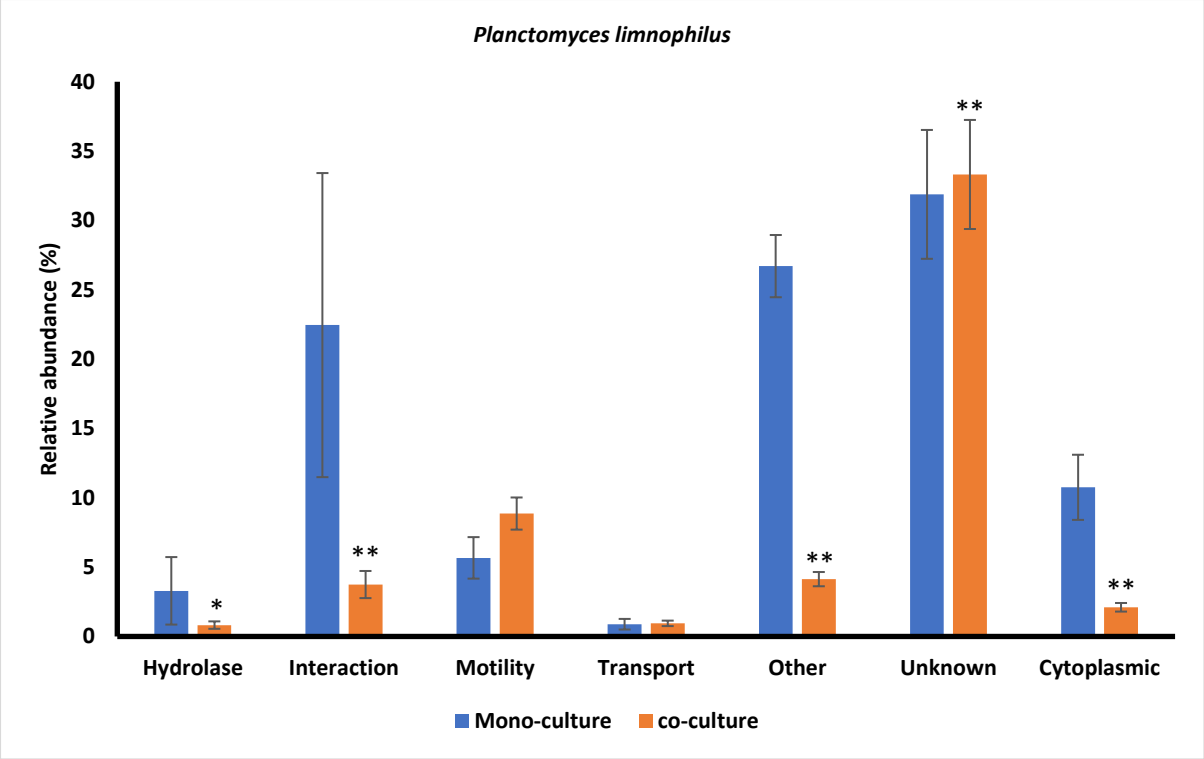

Figure 4.2.1.5.1: Functional category abundance of proteins found in the exoproteome of *P. limnophilus* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.5.1: List of up and down-regulated proteins of *P. limnophilus* of co-culture vs mono-culture.

Accession no	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
YP_003631568.1	hypothetical protein Plim_3556 (Rossmann-fold NAD(P)(+)-binding proteins)	Unknown	8.7	0	25.61	>0.1
YP_003631758.1	FKBP-type peptidylprolyl isomerase	Other	1.7	0.45	1.20	Non-classical secretion
YP_003631371.1	flagellin domain-containing protein	Motility	1.6	0.13	8.86	Non-classical secretion
YP_003628430.1	polyribonucleotide nucleotidyltransferase	Cytoplasmic	1.3	0.76	0.95	>0.1
YP_003628354.1	hypothetical protein Plim_0304	Transport	1.1	0.84	0.94	Non-classical secretion
YP_003628506.1	hypothetical protein Plim_0457	Cytoplasmic	-19.4	0	0.05	Non-classical secretion
YP_003630665.1	xylose isomerase	Cytoplasmic	-38.4	0	0.19	>0.1
YP_003629564.1	oxidoreductase domain-containing protein	Other	-54.0	0	0.07	Non-classical secretion
YP_003628853.1	hypothetical protein Plim_0809 (NHL repeat unit of beta-propeller proteins. The repeats have a catalytic activity in Peptidyl-glycine alpha-amidating monooxygenase)	Other	-67.8	0	0.06	Signal peptide
YP_003632271.1	hypothetical protein Plim_4265	Unknown	-75.8	0	0.02	Non-classical secretion

4.2.1.5.2 *Rhodopirellula baltica* SH1: The total number of proteins detected in *R. baltica* SH1 was as low as 17. In *R. baltica*, only one alkaline phosphatase was detected which was 0.77% in mono-culture and 0.64% in co-culture (figure 4.2.1.5.2). Genome analysis of *R. baltica* revealed a large number of sulfatases which helps to utilize carbon from complex sulfated heteropolysaccharides (Glockner et al., 2003). *R. baltica* also contains a large number of CAZymes. The lifestyle of *R. baltica* reveals that it can attach to and can degrade marine snow particles and CAZymes might play a role in the degradation of these particles (Wecker et al., 2010). Nevertheless, the exoproteome analysis here does not reveal a high abundance of hydrolytic enzymes. Proteins involved in interaction and membrane transport were more abundantly detected. Interestingly, some proteins possibly involved in cell adhesion were much more abundantly detected in co-culture (table 4.2.1.5.2). Flagellin proteins and, hence, motility were also abundantly detected in *R. baltica*. Previous studies revealed that the life cycle of *R. baltica* consists of aggregate forming sessile forms and a motile swarmer cell (Glockneret al., 2003).

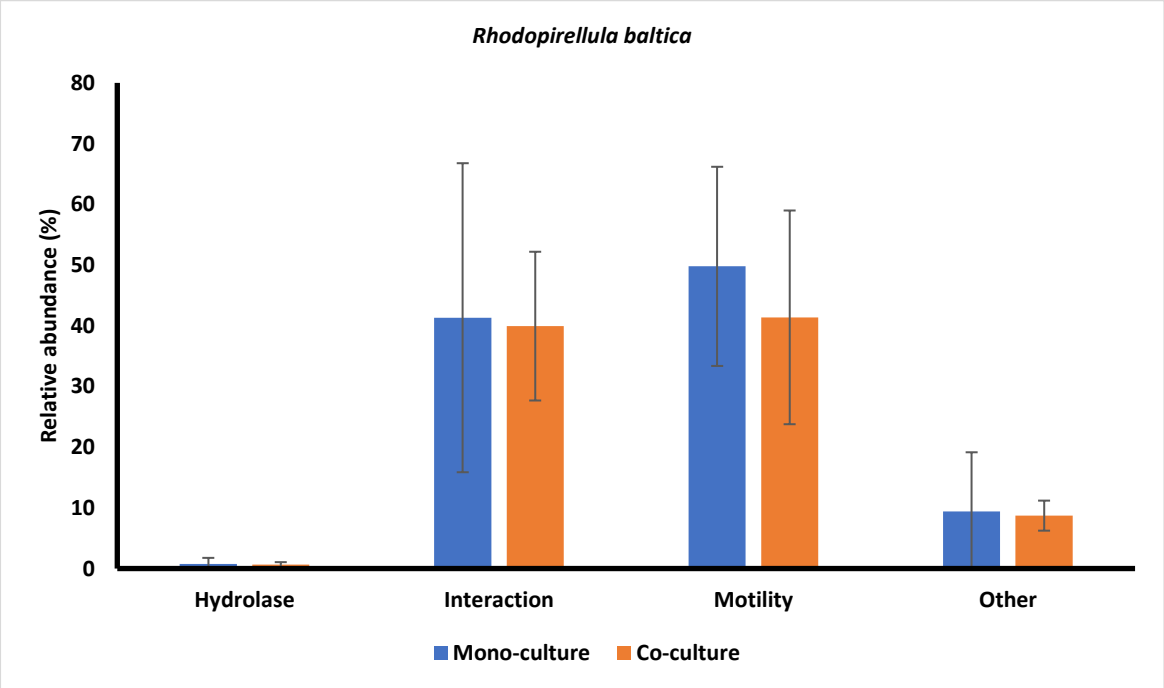

Figure 4.2.1.5.2: Functional category abundance of proteins found in the exoproteome of *R. baltica* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. (no asterisk: no protein within the pathway was significantly differentially detected).

Table 4.2.1.5.2: List of up and down-regulated proteins of *R. baltica* of co-culture vs mono-culture.

Accession no	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
NP_864105.1	surface-associated protein cshA precursor	Interaction	23.3	0.18	6.48	Non-classical secretion
NP_867765.1	surface-associated protein cshA precursor	Interaction	18.3	0.09	8.95	Non-classical secretion
NP_865314.1	cyclic nucleotide-binding protein	Interaction	6.4	0.13	4.32	Non-classical secretion
NP_864946.1	hypothetical protein RB2442 (Cadherin repeat-like domain ;Cadherins are glycoproteins involved in Ca2+-mediated cell-cell adhesion.)	Interaction	5.1	0.37	1.40	>0.1
NP_868682.1	hypothetical protein RB9053	Interaction	4.1	0.36	0.96	Non-classical secretion
NP_864127.1	hypothetical protein RB886 (Cadherin tandem repeat domain ;Cadherins are glycoproteins involved in Ca2+-mediated cell-cell adhesion.)	Interaction	-1.1	1	0.82	Non-classical secretion
NP_869169.1	NADH-dependent dyhydrogenase	Other	-1.1	0.96	0.37	>0.1
NP_866107.1	flagellin	Motility	-1.4	0.67	35.46	Non-classical secretion
NP_865206.1	signal peptide	Interaction	-48.7	0.20	1.01	Signal peptide

4.2.1.6 Verrucomicrobia: Like Planctomyces, Verrucomicrobia is also a largely unknown group of organisms that is recently gaining relevance due to a number of studies that suggest its involvement in a number of relevant processes (Lee et al., 2009). The total number of proteins detected in the exoproteome of *Verrucomicrobiae* bacterium was 358. Apart from the high abundance of cytoplasmic proteins, hydrolases were abundantly detected as seen in figure 4.2.1.6.1. A phosphoesterase was highly up-regulated in co-culture (87x fold) suggesting an important role in remineralising organic phosphorous produced by the phototroph. Phosphoesterases catalyse the hydrolysis of phosphoric acid. Interestingly, the pectate lyase WP_008099692.1 decreased its abundance in co-culture (15.4x fold) as opposed to what was observed in *R. pomeroyi* (above).

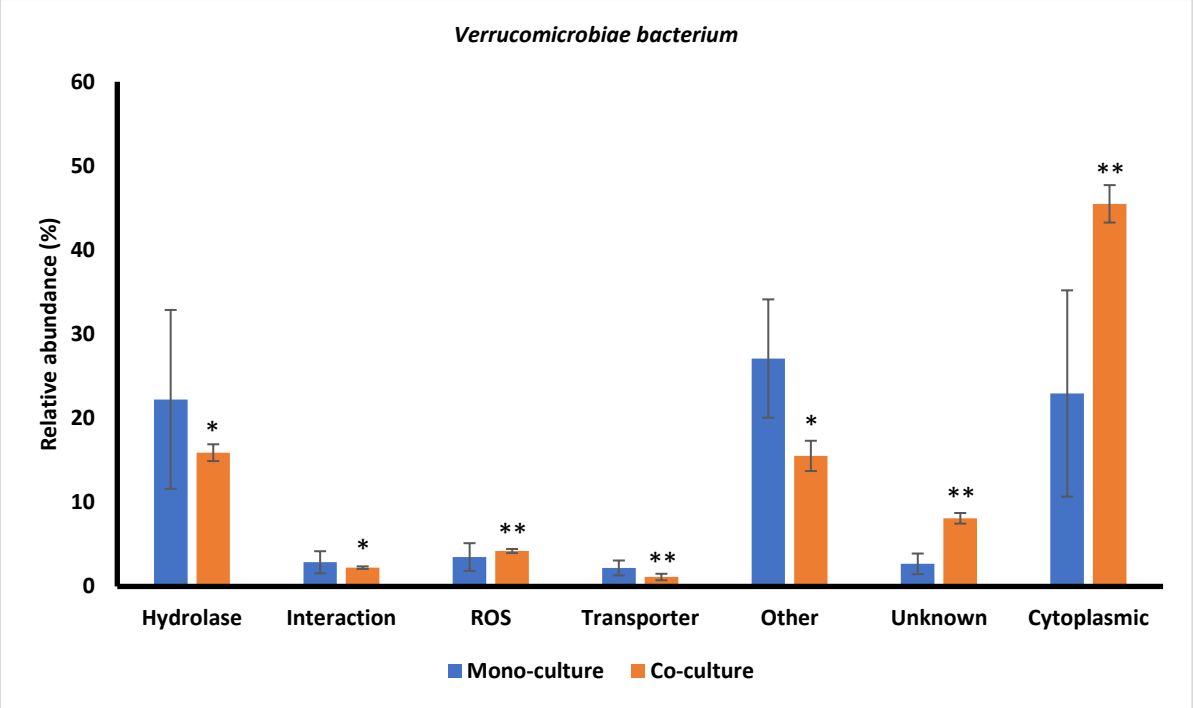

Figure 4.2.1.6.1: Functional category abundance of proteins found in the exoproteome of *V. bacterium* in mono-culture (blue bars) and when grown in co-culture with *Synechococcus* WH7803 (orange bars). Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

Table 4.2.1.6.1: List of up and down-regulated proteins of *V. bacterium* of co-culture vs mono-culture.

Accession no	Description	General category	Fold change (co-culture vs mono-culture)	q-value	% in co-culture	Rationale for selection
WP_008102145.1	phosphoesterase	Hydrolase	87.1	0	1.29	>0.1
WP_008097553.1	Oxidoreductase family, NAD-binding Rossmann fold protein	Other	66.5	0	1.37	Non-classical secretion
WP_008100025.1	Outer membrane protein	Other	46.2	0	1.10	Signal peptide
WP_008099606.1	nitrogen regulatory protein P-II 1	Cytoplasmic	45.1	0	0.63	>0.1
WP_008100737.1	hypothetical protein	Unknown	42.4	0	0.44	Non-classical secretion
WP_008099692.1	hypothetical protein (Pectate lyase superfamily protein)	Hydrolase	-15.4	0	0.02	Signal peptide
WP_008098625.1	GMC family oxidoreductase	Other	-18.1	0	0.00	Non-classical secretion
WP_008097378.1	auxiliary transport protein, MFP family, putative	Transporter	-19.7	0	0.00	Non-classical secretion
WP_008105414.1	Cna protein B-type domain	Interaction	-25.0	0	0.04	Non-classical secretion
WP_008100913.1	hypothetical protein	Unknown	-56.4	0	0.00	>0.1

4.2.2 Exoproteome analysis of Synechococcus WH7803

***4.2.2.1 PCA of the exoproteome of Synechococcus* sp. WH7803.** The PCA of the exoproteome of *Synechococcus* in co-culture with different heterotrophs when compared to axenic *Synechococcus* showed that the presence of most heterotrophs does not induce a large variation of the exoproteome of the phototroph (i.e. heterotrophs *A. marinum*, *Polaribacter sp., G. forsetii*, *F. agariphila*, *R. pomeroyi*, *R. denitrificans*, *D. shibae*, *M. adhaerens*, *P. stutzeri*, *R. baltica* and *V. bacterium*) whereas heterotrophs *S. tropica*, *A. machipongonensis*, *P. citrea*, *A. macleodii* and *P. limnophilus* did show a stronger influence on *Synechococcus* exoproteome (Figure 4.2.2.1).

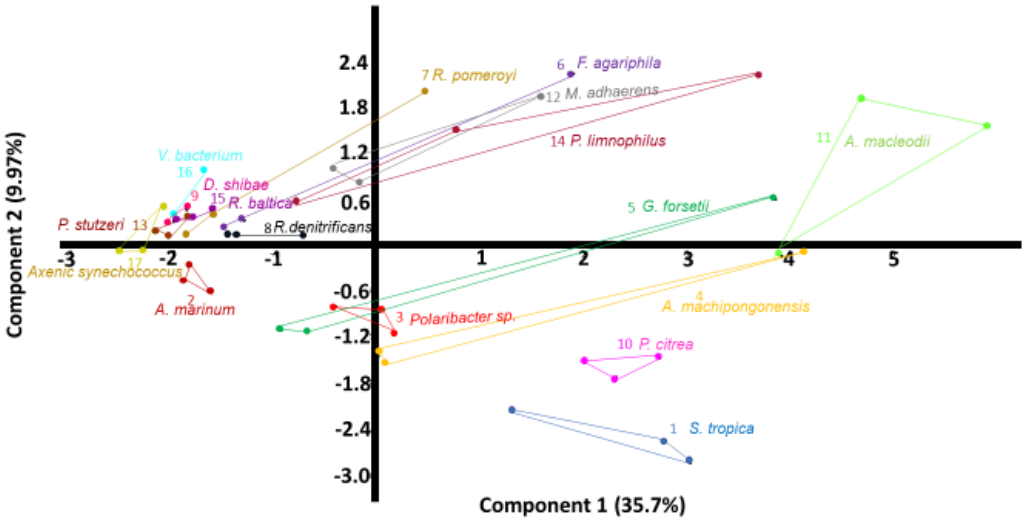

Fig. 4.2.2.1. PCA of the normalized exoproteomes of *Synechococcus* sp. WH7803 in a co-culture with 16 different heterotrophs and axenic *Synechococcus* WH7803

4.2.2.2 Categories of Synechococcus WH7803 *in co-culture with different heterotrophs*

The functional categories for proteins found in the exoproteome of *Synechococcus* were as analysed in chapter 3. Figure 4.2.2.2 represents the average abundance of the categories of *Synechococcus* in a co-culture with the 16 different heterotrophs in comparison to *Synechococcus* grown axenically. Generally, the relative abundance of membrane

transporters was lower in the exoproteome of *Synechococcus* when grown in co-culture except in the presence of *Aeromicrobium* which was higher. Mainly, these membrane transporters are for the uptake of inorganic nutrients. The downregulation of transporters observed in the presence of most heterotrophs may come as a consequence of the higher nutrient cycling when the heterotroph is present and, hence, a higher availability of nutrients that requires a lower investment in the biosynthesis of costly transporters as previously suggested (Christie-Oleza et al., 2017; Saito et al., 2014). The relative abundance of other cellular processes and of oxidative stress was higher when *Synechococcus* was grown in co-culture as compared to the axenic culture, again, except for the co-culture with *Aeromicrobium*. Cyanobacteria are thought to depend on heterotrophic organisms to deplete the ROS from the media, because most marine cyanobacteria lack catalase (Hunken et al., 2008; Morris et al., 2008; 2011). Nevertheless, *Synechococcus* sp. WH7803 does encode for all mechanisms required to deal with oxidative stress. The high abundance of these proteins in this analysis, even in the presence of heterotrophs, proves that bacteria may not be quenching all the ROS believed and that phototroph-heterotroph interactions are built on nutrient cycling more than on ROS quenching. As previously reported, DOM produced by *Synechococcus* is not only made of carbohydrates (Biersmith and Benner, 1998; Bertlisson et al., 2005), but also from proteins (Christie-Oleza et al., 2015a; 2017). Proteins involved in photosynthesis, but mainly form the photosynthetic antenna, are always highly detected in the exoproteome of *Synechococcus* (Christie-Oleza, 2015), a phenomenon that is difficult to explain.

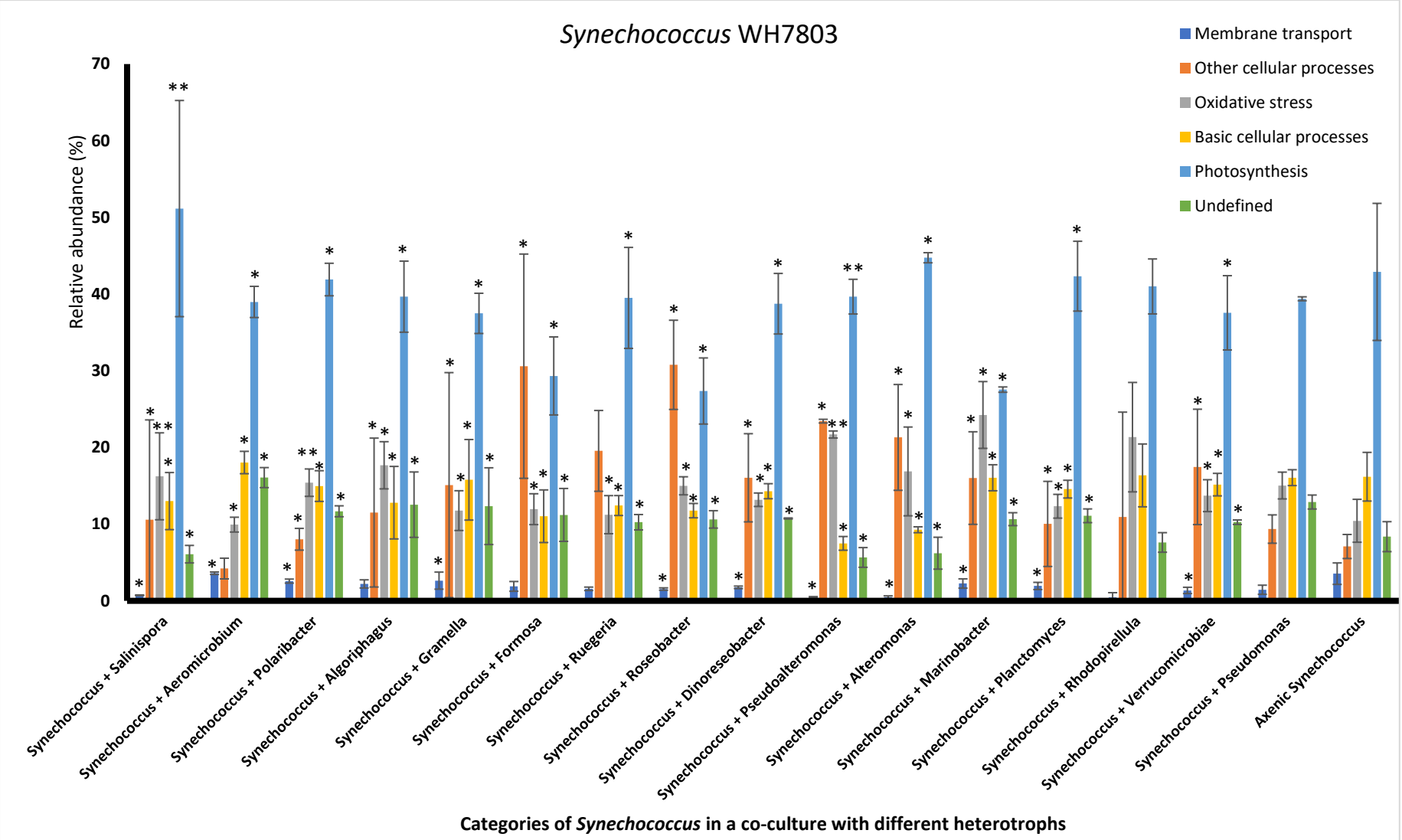

Fig. 4.2.2.2. Relative abundance of categories of *Synechococcus* WH7803 in a co-culture with different heterotrophs and of axenic culture of *Synechococcus*. Error bars represent the standard deviation of three independent replicates. Asterisks represent significant changes (no asterisk: no protein within the pathway was significantly differentially detected; one asterisk: less than 50% of the proteins that made up the pathway were significant; two asterisks: over 50% of the proteins were significant).

4.3 Conclusion

This analysis of 16 different heterotrophs in a mono-culture and co-culture has produced important data about marine phototroph-heterotroph interactions and their secreted proteins. Some heterotrophs seem to respond more extensively and through different means to the presence of *Synechococcus* sp. WH7803. Differentially expressed proteins involved in hydrolytic activities, motility and membrane transport in the presence of the phototroph *Synechococcus* sp. WH7803 suggests some microbes are better adapted than others to the interaction with this cyanobacterium. The data generated has highlighted interesting exoenzymes and substrate exchanges that require further investigation. For example, the upregulation of alpha amylases in a number of heterotrophs suggests *Synechococcus* is producing some kind of polysaccharide or oligosaccharide that induce these kinds of proteins. Alpha amylase was highly upregulated in the exoproteome of *S. tropica*, but S*ynechococcus* do not produce starch. This suggests that alpha amylase degrades some other polysaccharide or oligosaccharide which are unknown. This requires further research. It has been detected previously that *A. marinum* do not degrade starch, cellulose, chitin and casein, and it was proved in this analysis. But, *A. marinum* has been proved to degrade proteins, lipids and polysaccharides in this project. General trends have been observed at a larger taxonomic level. Hence, while Roseobacter strains show a high abundance in active membrane transporter in order to uptake organic substrates produced by the phototroph, other groups show a higher production of hydrolytic enzymes with the objective to breakdown the DOM. *R. pomeroyi* and *R. denitrificans* show a high abundance in active membrane transporter as compared to *D. shibae*, which show a high production of hydrolytic enzymes. This proved that Roseobacters are able to degrade the DOM produced by phototrophs. Also, CFB group was confirmed as specialised degraders of biopolymers such as polysaccharides (Reichenbach et al., 1991) as suggested by the abundance of hydrolytic enzymes detected during this analysis. Other groups such as the Gammaproteobacteria also show a large array of hydrolytic enzymes but for a higher preference of nitrogen-rich compounds as previously suggested

(Muhlenbruch et al., 2018). The abundant detection of proteins involved in motility or attachment also suggests an interesting characterisation of the preferred lifestyle of each heterotroph when grown in the presence of *Synechococcus.* The reduced number of proteins detected in Planctomycete strains suggests the culture conditions were not optimal.

Chapter 5

Characterisation of proteins secreted by *R. pomeroyi* DSS-3 and their role in the interaction with *Synechococcus* sp. WH7803

5.1 Introduction

Degradation of different polysaccharides by marine microbes has been identified in different marine microbes. Algal polysaccharides are hydrolysed by glycoside hydrolases and polysaccharide lyases present in the CAZyme database (Lombard et al., 2014). The structural and functional characterisation of various marine CAZymes revealed the mechanisms that helps to degrade polysaccharides of red, brown and green seaweed i.e. agar, carrageenan, alginate and ulvan polysaccharides (Hehemann et al., 2014). Marine flavobacterium *Zobellia galactanivorans* was used as a model organism to study the degradation of algal polysaccharides. Genome analysis of *Z. galactanivorans* revealed that it contains the highest number of genes for glycoside hydrolases, polysaccharide lyases and carbohydrate esterases and the second highest number of sulfatase genes as compared to 125 other marine heterotrophic bacteria genomes, which allows this organism to degrade a large array of algal polysaccharides (Barbeyron et al., 2016). It has been reported that alginate is one of the most abundant marine algal polysaccharide which accounts for more than 50% of the dry weight of the marine brown macroalgae (Mabeau and Kloareg, 1987). Alginate acts as a nutrient source for different heterotrophic bacteria (Thomas et al., 2012). The genome analysis of the marine bacterium *Alteromonas macleodii* 83-1 (strain used in Chapter 4) revealed that it contains an alginolytic system that comprises 5 alginate lyases being the mechanism behind its ability to degrade the alginate and other algal polysaccharides. *Alteromonas macleodii* 83-1 can also degrade laminarin, pullulan and xylan as suggested by its encoded polysaccharide utilization loci in its genome (Neumann et al., 2015). Laminarin is another abundant polysaccharide present in algal organic matter and the degradation of this polysaccharide involves two laminarinase enzymes i.e. endo- and exo-acting enzymes (Becker et al., 2017). Metagenomic and proteomic data on marine algal blooms revealed specific ecologically relevant enzymes and supported the fact that laminarin can be used as a source of carbon and energy for heterotrophs (Teeling et al., 2012). In Bacteroidetes, glycans are degraded by CAZYmes that are localised in polysaccharide utilization loci (Martens et al., 2009, Tancula et al., 1992). PUL systems to degrade the laminarin has also been identified in *F. agariphila* (Mann et al., 2013).

Two pectate lyases have also been detected in marine microbes but, interestingly, they are believed to have been acquired from terrestrial origin (Hehemann et al., 2017). The pectin specific pathway was first detected in the marine bacterium *Pseudoalteromonas haloplanktis* (Truong et al., 2001) and proteomic experiments revealed that this marine microbe releases pectate lyase into the environment in the presence of pectin (Hehemann et al., 2017). The detection of pectin degradation pathway in *P. haloplanktis* indicates a new substrate niche for marine microbes. Pectin is abundantly synthesized by terrestrial plants (Mohnen, 2008). Pectin has also been identified in red algae, green algae, seagrasses and in marine microalgae (Popper et al., 2011) although most sources of pectin in the marine environment are believed to come from trees or plants that enter the oceans from coastal runoff and through rivers (Hehemann et al., 2017). The detection of pectin related CAZymes in environmentally abundant marine *Alteromonas* and *Pseudoalteromonas* strains supports the fact that pectin may well be an ecologically relevant substrate in the oceans (Hehemann et al., 2017). *Alteromonas macleodii* 83-1 has shown a biphasic phenotype in which it first utilizes laminarin and then it is followed by simultaneous utilization of alginate/pectin (Koch et al., 2018). This provides molecular evidence that the biodegradation of macroalgae polysaccharide mixtures may have cellular adaptations and preferences for certain substrates over others, allowing for more complex niche partitioning in microbial communities. While there has been a considerable amount of research carried out on glycosidases, other hydrolytic functions for polymeric substances present in the photosynthate, such as proteases and lipases, has been less characterized in marine heterotrophs. Alkaline proteases are also important enzyme that play a vital role in making phosphorous available to the microbial community. One of the alkaline proteases produced by marine *Pseudoalteromonas* sp. 129-1 was purified and characterized, with a further potential use in detergents and anti-biofilm agents (Wu et al., 2015). Some proteases have been found in deep-sea bacteria, for example, myroilysin from *Myroides profundi* D25 (Chen et al., 2009) and pseudoalterin from *Pseudoalteromonas* sp. CF6-2 (Zhao et al., 2012). These extracellular proteases play important roles in particulate organic nitrogen degradation by breaking down high molecular weight organic nitrogen to

LMW dissolved organic nitrogen (Zhao et al., 2012, Chen et al., 2009). A new M13 metallopeptidase from deep-sea *Shewanella* sp. E525-6 was characterized, which helps to understand marine bacterial peptidases and degradation of organic nitrogen (Yang et al., 2016).

In Chapter 3, the exoproteomic analysis of *R. pomeroyi* DSS-3 in a co-culture with *Synechococcus* WH7803 revealed some interesting exoenzymes which are produced by *R. pomeroyi* to degrade the DOM of *Synechococcus*. Nevertheless, the function of these exoenzymes is poorly characterised.

The hypothesis of this PhD thesis is that marine phototroph-heterotroph interactions are mostly based on nutrient recycling (Christie-Oleza et al., 2017) and exoenzymes play an important role within this process by degrading the DOM of phototrophs. The potential exoenzymes that may play an important role in these interactions have been identified by exoproteomics in chapters 3 and 4 of this thesis. Unfortunately, most of these proteins are currently poorly characterized and their function has only been inferred from annotation pipelines that, in many cases, are based on the similarity of domains within the proteins. Hence, the annotated functions are not always correct. The main aim of this chapter was to further characterise these proteins (mainly, hydrolytic-like enzymes identified in Chapter 3) and to determine their potential role in marine nutrient cycling. This chapter also aimed to study the substrates for some of these hydrolytic enzymes. The two most abundantly detected hydrolytic enzymes in the exoproteome of *R. pomeroyi* in the presence of *Synechococcus* were presumably a pectate lyase and sialidase-like enzyme (Kaur et al., 2017), although these remain annotated as hypothetical proteins in the genome. Some previous analyses also revealed some other interesting hydrolytic enzymes that were identified after a seven-day co-culture experiment (Christie-Oleza et al., 2015) and, hence, these were also included in the analysis performed in this chapter. Pectate hydrolase mutant in this project was tested with different substrates i.e. pectin, starch, alginate, cellulose and laminarin. Sialidase mutant was

also tested with NANA to characterise it. The methods for these growth experiments have been discussed in chapter 2 of materials and methods under section 2.5.

5.1.1 Rationale behind the selection of the secreted hydrolytic enzymes and interaction proteins of R. pomeroyi used in this chapter: The hydrolytic enzymes and interaction proteins (Figure 5.1.1) presumably involved in microbial interactions were selected mainly based on the findings from chapter 3 (Kaur et al., 2018). Interestingly, most of these proteins were originally annotated as 'hypothetical proteins' and a plausible hydrolytic function could be given only after a bioinformatic search. The average relative abundance of these secreted proteins in two different media, i.e. in ASW and in SW, were extracted from the data obtained in chapter 3 and represented in table 5.1.1.

5.1.1.1. Potential hydrolytic proteins: Protein AAV93776.1, initially annotated as a hypothetical protein, had functional domains similar to pectate lyases as shown by a Conserved Domain search (CDS function in NCBI). The average abundance of this protein was 2.13% in ASW and 2.68% in SW but with strong variations over the 100-day time course experiment (Table 5.1.1). In the Roseobacter clade, this CDS is highly conserved (Christie-Oleza, 2015). Pectate lyases can degrade the pectin which is present in the primary cell wall of higher plants (Carpita and Gibeaut, 1993). It has been detected in the marine micro-organisms *Gramella forsetii* and *Gramella flava* that they can degrade and utilize pectin as a source of carbon and energy (Tang et al., 2017). In *G. flava*, polysaccharide utilization loci encoding for the enzymes involved in degrading pectin are identified, although the pectin degradation mechanism is unclear. Proteomic analysis of the marine *P. haloplanktis* ANT/505 also revealed that it produces two pectate lyases, i.e. PelA and PelB, in response to the pectin which is present in the environment (Hehemann et al., 2017). *P. haloplanktis* has a complete pathway for pectin degradation which shows that for this marine microbe pectin acts as a new substrate niche (Hehemann et al., 2017).

The other potential hydrolase, AAV95476.1, has some homology domains with sialidase enzymes as previously described (Christie-Oleza et al., 2015). On average, it was 0.91%

abundant in ASW and 1.35% in SW but also showed large variations throughout the 100-day co-culture experiment. Sialidases are involved in the binding and hydrolyzation of terminal sialic acid residues from various glycoconjugates. They play vital roles in pathogenesis, bacterial nutrition and cellular interactions (Rothe et al., 1991).

AAV96145.1 is another hydrolase as inferred by bioinformatics analyses, containing similar domains to Ser/Thr phosphatase/nucleotidase proteins. In the 100-day time course experiment, the average abundance of this protein in ASW was 0.4% and 0.59% in SW, and showed much smaller variation throughout the 100 days of incubation (Table 5.1.1). It was searched in NCBI on the basis of its conserved domain as ecto-5'-nucleotidase (ecto-5'-NT). The role of ecto-5'-NT is to hydrolyse AMP to adenosine and phosphate (Norbert Strater, 2006). But the exact function in *R. pomeroyi* remains unclear.

AAV96272.1 was annotated as metallo-beta-lactamase family protein. Its average abundance was 0.21% in ASW and 0.27% in SW. Metallo-β-lactamases are important enzymes involved in the biodegradation of different β-lactam drugs such as carbapenems (Palzkill, 2013) but, again, its role in *R. pomeroyi* is unknown.

AAV97448.1 was annotated as amidohydrolase with average abundance 0.14% in both ASW and SW 100-day time course experiment. Presumably, this amidohydrolase is involved in the hydrolysis of amide bonds.

AAV95890.1 is a serine protease and its abundance was 0.22% in ASW and 0.03% in SW (table 5.1.1). Its role in protein hydrolysis needs further investigation.

AAV95139.1 is a PhoX alkaline phosphatase. It made up 0.46% of *R. pomeroyi*'s exoproteome in ASW and 0.27% in SW. The function of PhoX was previously confirmed (Sebastian and Ammerman, 2011). Annotated as a twin-arginine translocation pathway signal sequence domain protein, this alkaline phosphatase is presumably exported folded across the cytoplasmic membrane of bacteria (Sargent et al., 2005). PhoX is the prevalent alkaline phosphatase form used by marine microbes to acquire organic phosphorus in the ocean.

5.1.1.2. Proteins involved in interaction: AAV93680.1 and AAV95658.1 are RTX toxin related proteins which were abundantly detected in the exoproteome of *Roseobacter* isolates (Christie-Oleza et al., 2010; Christie-Oleza et al., 2012) and which are presumably involved in microbial interactions. The RTX toxin family are a group of cytotoxins produced by Gram negative bacteria, although the cytotoxicity of these proteins possibly does not hold true for all proteins annotated with this function. The RTX family is defined by two common features: characteristic repeats in the protein sequences, and extracellular secretion by the type 1 secretory system. It has glycine and aspartate-rich repeats located at the C-terminus of the toxin proteins (Linhartova, Bumba et al., 2010). The enormous diversity of sequences found within this group and additional functional domains that co-occur on these proteins suggests they may have alternative roles such as adhesion. AAV93552.1 is another RTX-like protein encoded by *R. pomeroyi* and annotated as PaxA. PaxA was previously detected in the exoproteome of *R. pomeroyi* in which it represented over 50% of the total exoproteome in rich marine broth medium (Christie-Oleza and Armengaud, 2010). During the 100-day time course experiment the average abundance of this protein was much lower, i.e. 0.62% in ASW and 0.03% in SW.

AAV93800.1 is suggested to be a microcystin dependent protein that might play an important role in plant-microbe interactions as shown by a bioinformatics analysis. The average abundance of this protein was 0.14% in ASW and 8% in SW in time course experiment.

AAV96685.1 is *R. pomeroyi*'s flagellin and major component of the flagella. When *R. pomeroyi* induces a motile lifestyle, this protein is abundantly found in the exoproteome of *R. pomeroyi* as discussed in chapter 3. It was highly abundant in the exoproteome of *R. pomeroyi* with an average abundance of 23.2% in ASW and 35.0% in SW with enormous variations over the 100-day time course incubation with *Synechococcus*. *R. pomeroyi* can switch between a motile and a non-motile life form depending on the availability of nutrients (Kaur et al., 2018).

AAV95529.1 is the major capsid protein of the GTA encoded by *R. pomeroyi*. GTAs are produced by a large variety of bacteria which carry random fragments form the host's genome

and that are thought to encourage horizontal gene transfer (Frost et al., 2005). GTAs are highly conserved in the Roseobacter clade (Zhao, Wang et al. 2009). These bacteriophage-like particles involved in the exchange of genetic material are abundantly detected in the exoproteome of *R. pomeroyi*, although their role when interacting with *Synechococcus* is unclear.

Table 5.1.1: The average of relative abundance of the hydrolytic and interaction proteins from different time points in ASW and SW.

Accession no.	Mutant no.	Possible function	Media	Day 1[a]	Day 3[a]	Day 7[a]	Day 14[a]	Day 21[a]	Day 32[a]	Day 60[a]	Day 100[a]	Average
AAV93776.1	1	Pectate lyase	ASW	4.94	4.05	3.23	3.08	0.04	0.02	0.08	1.63	2.13
			SW	1.43	1.88	2.16	2.29	3.08	3.12	3.84	3.61	2.68
AAV93680.1	2	RTX-like	ASW	1.44	0.61	0.67	0.73	0.00	0.00	0.00	0.52	0.50
			SW	0.31	0.25	0.22	0.18	0.09	0.06	0.03	0.02	0.15
AAV95476.1	3	Sialidase	ASW	1.14	1	0.99	1.35	0.8	0.43	0.51	1.07	0.91
			SW	1.07	1.08	1.31	1.22	1.77	1.59	1.49	1.25	1.35
AAV93800.1	4	Microcystin	ASW	0.01	0.02	0.03	0.03	0.24	0.31	0.23	0.22	0.14
			SW	0.09	0.07	0.22	0.28	0.10	3.35	3.12	4.35	8.00
AAV95658.1	5	RTX-like	ASW	0.35	0.31	0.29	0.51	0.02	0.02	0.02	0.42	0.24
			SW	0.29	0.28	0.37	0.39	0.28	1.23	2.24	2.68	2.35
AAV96145.1	6	Ser/Thr protein phosphatase/nucleotidase	ASW	0.04	0.05	0.27	0.13	0.93	0.77	0.7	0.34	0.4
			SW	0.06	0.18	0.23	0.44	0.69	0.58	1.02	1.49	0.59
AAV96272.1	7	metallo-beta-lactamase	ASW	0.06	0.11	0.12	0.22	0.38	0.26	0.28	0.22	0.21
			SW	0.03	0.1	0.23	0.28	0.35	0.37	0.38	0.43	0.27
AAV97448.1	8	Amidohydrolase	ASW	0.04	0.03	0.04	0.04	0.32	0.26	0.27	0.16	0.14
			SW	0.02	0.07	0.11	0.15	0.15	0.08	0.18	0.4	0.14
AAV95890.1	9	Protease	ASW	0.03	0.01	0.01	0.02	0.51	0.56	0.55	0.09	0.22
			SW	< 0.01	0.01	0.01	0.01	0.01	0.03	0.07	0.13	0.03
AAV95139.1	10	PhoX	ASW	0.58	0.56	0.4	0.33	0.16	0.88	0.65	0.15	0.46
			SW	0.53	0.54	0.57	0.28	0.13	0.08	0.02	< 0.01	0.27
AAV96685.1	11	Flagellin protein	ASW	43.85	50.83	54.62	22.13	0.15	0.24	0.90	12.89	23.20
			SW	45.52	53.30	43.47	38.41	31.73	33.52	21.84	12.71	35.06
AAV95529.1	12	GTA	ASW	1.14	1.19	0.56	0.93	0.00	0.00	0.01	0.27	0.51
			SW	0.74	0.54	0.58	0.59	0.54	0.38	0.31	0.12	0.48
AAV93552.1	13	PaxA	ASW	0.44	0.46	0.49	0.72	0.38	0.55	1.01	0.95	0.62
			SW	0.01	0.02	0.03	0.03	0.04	0.03	0.03	0.02	0.03

a. The values shown are the average of relative abundance obtained from three biological replicate cultures.

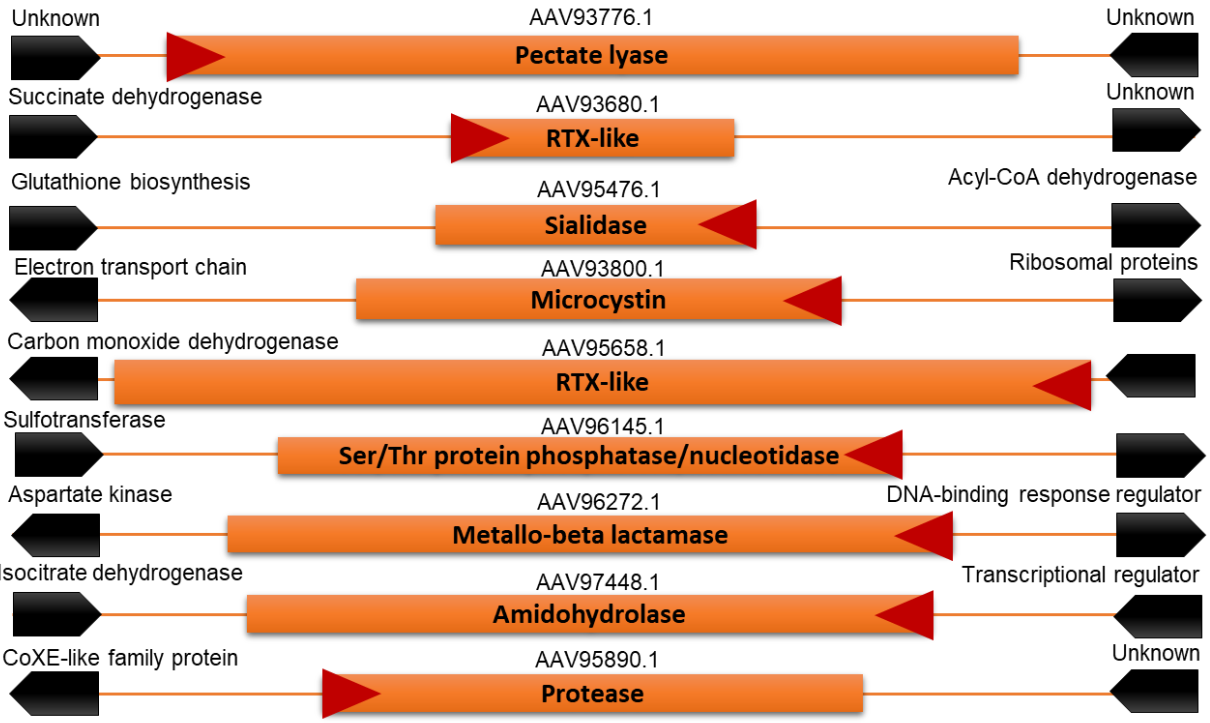

Figure 5.1.1: Hydrolytic enzymes and interaction proteins that were detected in the exoproteome of *R. pomeroyi* in a co-culture with *Synechococcus* WH7803 in 100-day time course experiment and were mutated. The annotated function was based on the conserved domain. Arrow indicates transcription orientation.

5.2 Results and discussion

5.2.1. Knockout mutants of R. pomeroyi: The different hydrolytic enzymes and interaction proteins listed in table 5.2.1.1. were identified in *R. pomeroyi* during its co-culture with *Synechococcus* (Chapter 3) plays a role in microbe-microbe interactions, and further characterisation of these hydrolytic enzymes and interaction proteins was the main aim of this chapter.

Knock-out mutants were generated in each one of the genes that encoded for these secreted proteins to i) further characterise their function, and ii) determine their role during the heterotroph's interaction with the *Synechococcus* sp. WH7803. Mutants 1 to 9 (table 5.2.1.1) were generated by the author, and figure 5.2.1.1 represents the size of the wild type (W) *R. pomeroyi* and the mutants (M). All of these mutants were confirmed by sequencing as mentioned in chapter 2. The knock-out mutant in *phoX* (mutant 10 in table 5.2.1.1) was

obtained from Dr Sebastian (Sebastian and Ammerman, 2011). Mutants for the flagella (mutant 11) and GTA (mutant 12) was generated by the senior research technician Maria Aguilo Ferretjans at the University of Warwick. Mutant for the toxin-like protein PaxA (mutant 13) was provided by Dr Christie-Oleza, the principal supervisor of this research project. Table 5.2.1.2 represents the expected size of genes disrupted by the antibiotic resistance cassette *versus* the size of the wild type gene as well as the antibiotic resistance cassette used to disrupt the gene.

Table 5.2.1.1: List of hydrolytic enzymes and interaction proteins that were detected in the exoproteome of *R. pomeroyi* in a co-culture with *Synechococcus* WH7803 in 100-day time course experiment.

New Annotation	Old annotation	Description	General category	Mutants	Mutants generated by
AAV93776.1	YP_165722.1	hypothetical protein SPO0459 (Pectate lyase)	Hydrolase	Mutant 1	Author
AAV93680.1	YP_165625.1	type I secretion target repeat protein (Ca2+-binding protein, RTX toxin-related)	Interaction	Mutant 2	Author
AAV95476.1	YP_167436.1	BNR/Asp-box repeat domain protein (Sialidase)	Hydrolase	Mutant 3	Author
AAV93800.1	YP_165745.1	microcystin dependent protein, putative	Interaction	Mutant 4	Author
AAV95658.1	YP_167620.1	type I secretion target repeat protein (Ca2+-binding protein, RTX toxin-related)	Interaction	Mutant 5	Author
AAV96145.1	YP_168112.1	Ser/Thr protein phosphatase/nucleotidase, putative	Hydrolase	Mutant 6	Author
AAV96272.1	YP_168240.1	metallo-beta-lactamase family protein	Hydrolase	Mutant 7	Author
AAV97448.1	YP_165143.1	amidohydrolase domain protein (plasmid)	Hydrolase	Mutant 8	Author
AAV95890.1	YP_167855.1	protease, S2 family	Hydrolase	Mutant 9	Author
AAV95139.1	YP_167097.1	twin-arginine translocation pathway signal sequence domain protein (PhoX)	Hydrolase	Mutant 10	Sebastian and Ammerman, 2011
AAV96685.1	YP_168655.1	flagellin protein	Interaction	Mutant 11	Maria Aguilo Ferretjans (University of Warwick)
AAV95529.1	YP_167489.1	portal protein, HK97 family (GTA)	Interaction	Mutant 12	Maria Aguilo Ferretjans (University of Warwick)
AAV93552.1	YP_165496.1	PaxA, putative	Interaction	Mutant 13	Joseph Christie-oleza (Principal supervisor of the project)

Table 5.2.1.2: List of knock-out mutants of *R. pomeroyi* displaying size of mutants, size of wild type and the antibiotics used for them.

Accession number	Mutants	Size of mutant (bp)	Size of wild type (bp)	Antibiotic used
AAV93776.1	1	2400	3769	Gm
AAV93680.1	2	1832	974	Gm
AAV95476.1	3	1860	1002	Gm
AAV93800.1	4	2400	2047	Gm
AAV95658.1	5	2400	4669	Gm
AAV96145.1	6	2400	2900	Gm
AAV96272.1	7	2400	3460	Gm
AAV97448.1	8	2400	3055	Gm
AAV95890.1	9	2400	2383	Gm

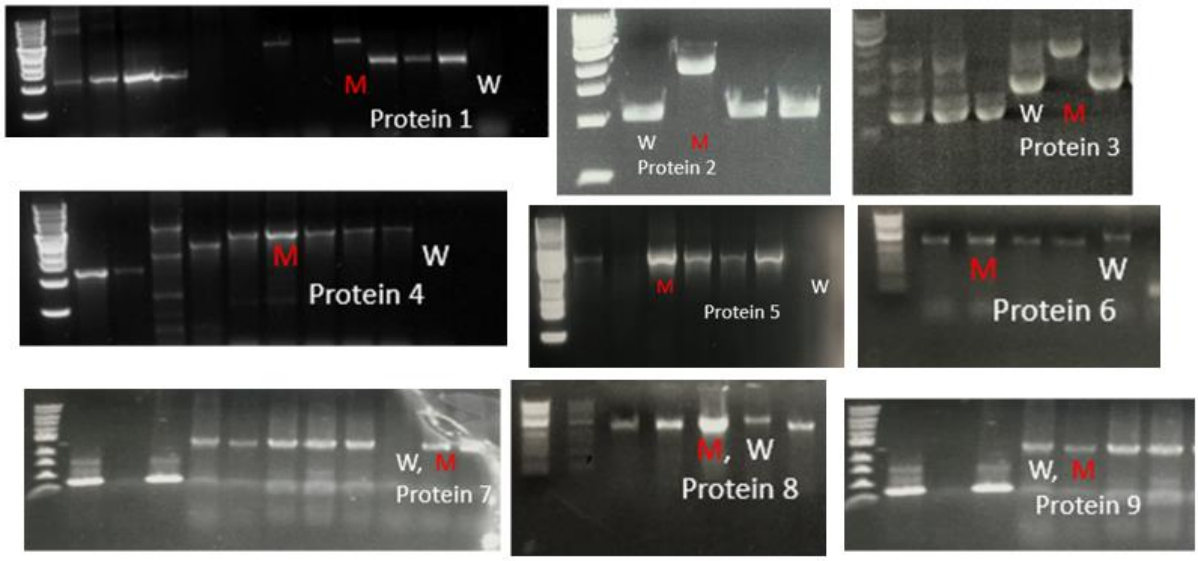

Figure 5.2.1.1: Gel images of mutants (M) 1-9 that shows their size as compared to wild type (W) *R. pomeroyi*. The mutants of expected size were confirmed by sequencing**.**

5.2.2 Characterisation of the mutants

5.2.2.1 Growth of the mutant 1 (pectate lyase) with different polysaccharide substrates

Pectate is a poly(1,4-α-D-galacturonate). The function assigned to AAV93776.1 was a pectate lyase, but with low confidence, hence, this protein may catalyse the hydrolysis of a different substrate. Furthermore, it is unlikely that *Synechococcus* produces pectin. The wild type *R.*

pomeroyi and the pectate lyase mutant were tested with a large range of different polysaccharides i.e. pectin, starch, alginate, cellulose and laminarin. The negative (no organic carbon source) and positive control (succinate) were also used for comparison. As seen in Figure 5.2.2.1.1, there were no differences between the wild type and the pectin lyase mutant amongst any of the conditions tested. The results suggest that *R. pomeroyi* is able to grow on the substrates pectate, alginate and laminarin but not on cellulose and starch. Unfortunately, from the work carried out it was not possible to determine the specific substrate for the protein AAV93776.1 and it could not be characterised as a pectate lyase. Another explanation is that *R. pomeroyi* has a large redundancy of functions meaning that, despite the knockout mutation, this heterotroph may have an alternative enzyme with similar function that can supplement AAV93776.1's activity. Further work would be required to investigate the molecular mechanism.

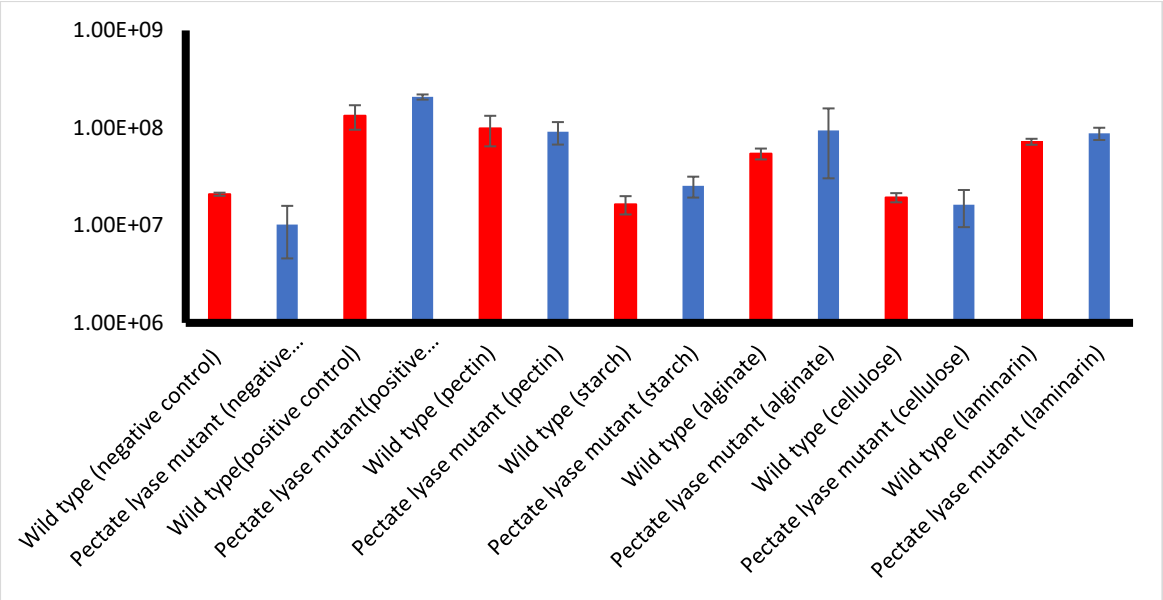

Figure 5.2.2.1.1: The growth of wild type (red) *R. pomeroyi* and pectate lyase mutant (blue) with different substrates over 3 days. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

5.2.2.2 Growth of the sialidase mutant with N-acetyl neuramic acid (NANA) and sialidate:

The sialidase-like mutant and wild type *R. pomeroyi* were grown with sialidate as well as the sialidate monomer NANA. We were expecting that both the sialidase mutant and wild type *R. pomeroyi* would degrade the NANA but there would be a difference between wild type and mutant in the presence of sialidate. Unfortunately, not even the wild type could grow using NANA as a source of carbon and energy as in this condition the cell abundance was even lower that when no source of carbon was added (negative control). Hence, more research is necessary to further characterise this enzyme.

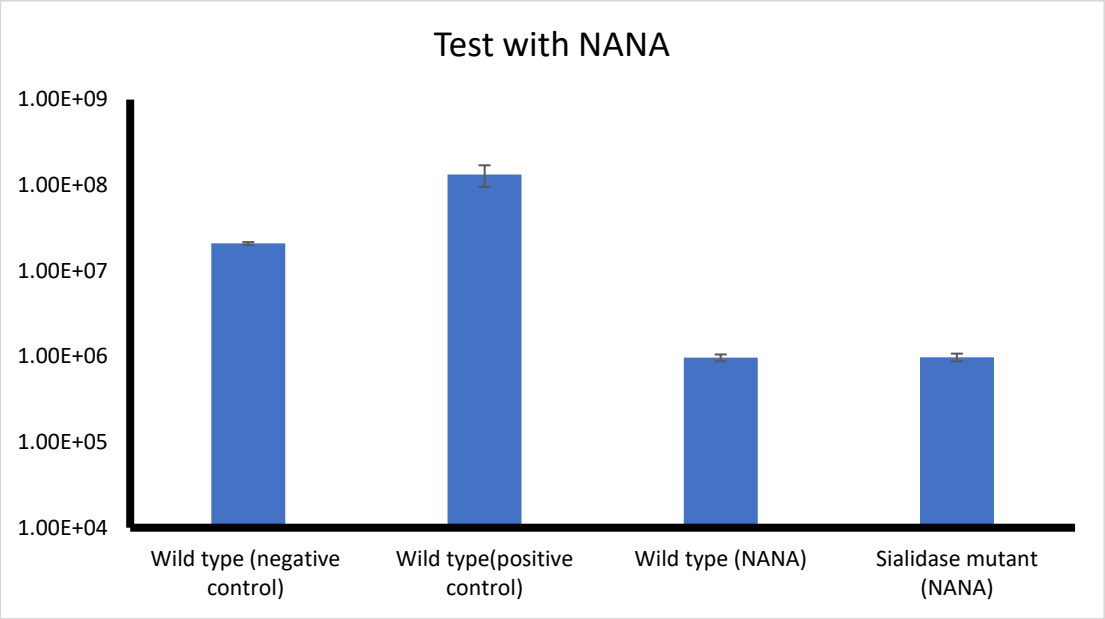

Figure 5.2.2.2.1: Growth of wild type *R. pomeroyi* and sialidase mutant on N-acetyl neuramic acid (NANA) over 3 days. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

5.2.2.3 Co-culture of wild type R. pomeroyi and mutants with Synechococcus during 100 days:

All mutants mentioned in Table 5.2.1.1 and a wild type *R. pomeroyi* were co-cultured with *Synechococcus* WH7803 for 100 days in both natural SW and ASW medium in order to explore if any of the mutations affected the positive *R. pomeroyi-Synechococcus* interaction observed previously (Christie-Oleza et al., 2017) due to the loss of the ability to remineralise

all compounds within the photosynthate. The cultures were sampled at different time points (days 0, 3, 7 14, 21, 37, 63, 78 and 100) in natural SW, but in ASW it was sampled for only 37 days because cultures got contaminated by second heterotrophic bacterium which produced yellow colonies. *R. pomeroyi* and *Synechococcus* survived together for up to 200 days but the axenic culture of *Synechococcus* died after just 4-6 weeks due to the accumulation of organic matter (Christie-Oleza et al., 2017). Here, in natural SW (Fig. 5.2.2.3.1), *Synechococcus* reached a steady state after day 21 in co-culture with all *R. pomeroyi* strains (wild type and mutants) showing almost identical growth patterns. Nevertheless, the axenic culture of *Synechococcus* looked different than when grown in co-culture as seen in the Fig. 5.2.2.3.1 (light grey line). The axenic *Synechococcus* showed a much less drastic initial drop in cell numbers at days 14 and 21, but had a stronger drop at day 100, suggesting the culture may have been in the process of being died. In natural SW, *R. pomeroyi* also reached a stable state after day 21 and the same pattern was seen in all strains except for mutants 10, 12 and 13 where cell abundance strongly declined at time point 78 (Fig. 5.2.2.3.2). The reason could be that they are not interacting productively with *Synechococcus* or there was insufficient DOM in a co-culture with the mutants 10,12 and 13 required for mutants to grow. Mutants grow less well therefore is inetersting. In ASW, growth of *Synechococcus* in a co-culture with different mutants and with *R. pomeroyi* showed the same pattern (Figure 5.2.2.3.3), and growth of *R. pomeroyi* and other mutants was similar except mutants 11 and 12 at time point 21 (Fig 5.2.2.3.4).

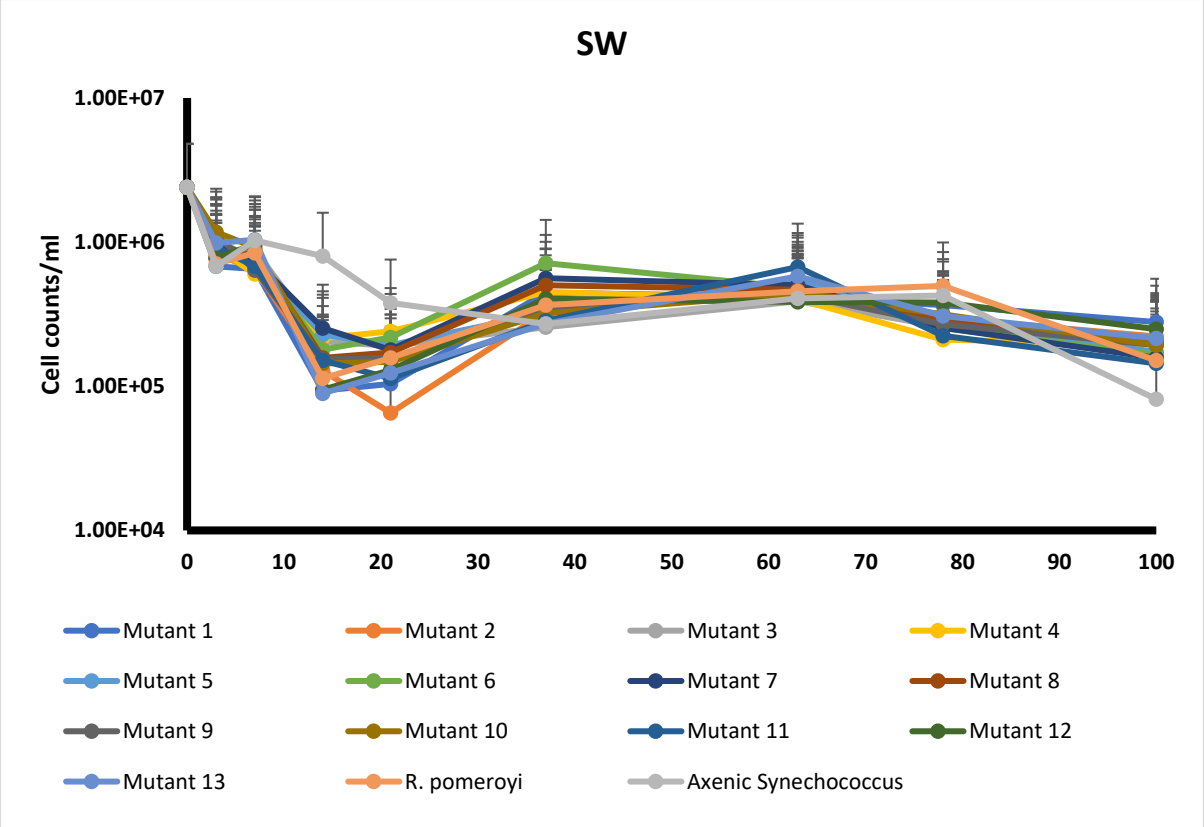

Fig. 5.2.2.3.1. Growth of *Synechococcus* WH7803 in a co-culture with different mutants of *R. pomeroyi* and wild type *R. pomeroyi* over the 100-day time course experiment in natural SW. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

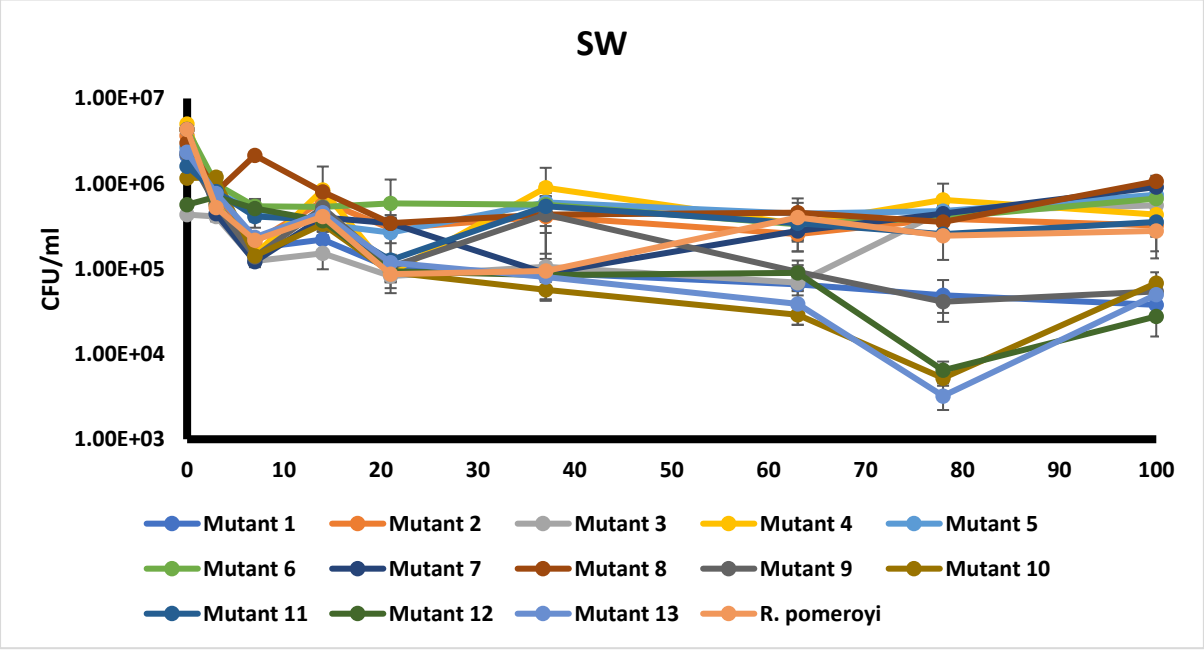

Fig. 5.2.2.3.2. Growth of different mutants of *R. pomeroyi* and wild type *R. pomeroyi* in a co-culture with *Synechococcus* WH7803 over the 100-day time course experiment in natural SW. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

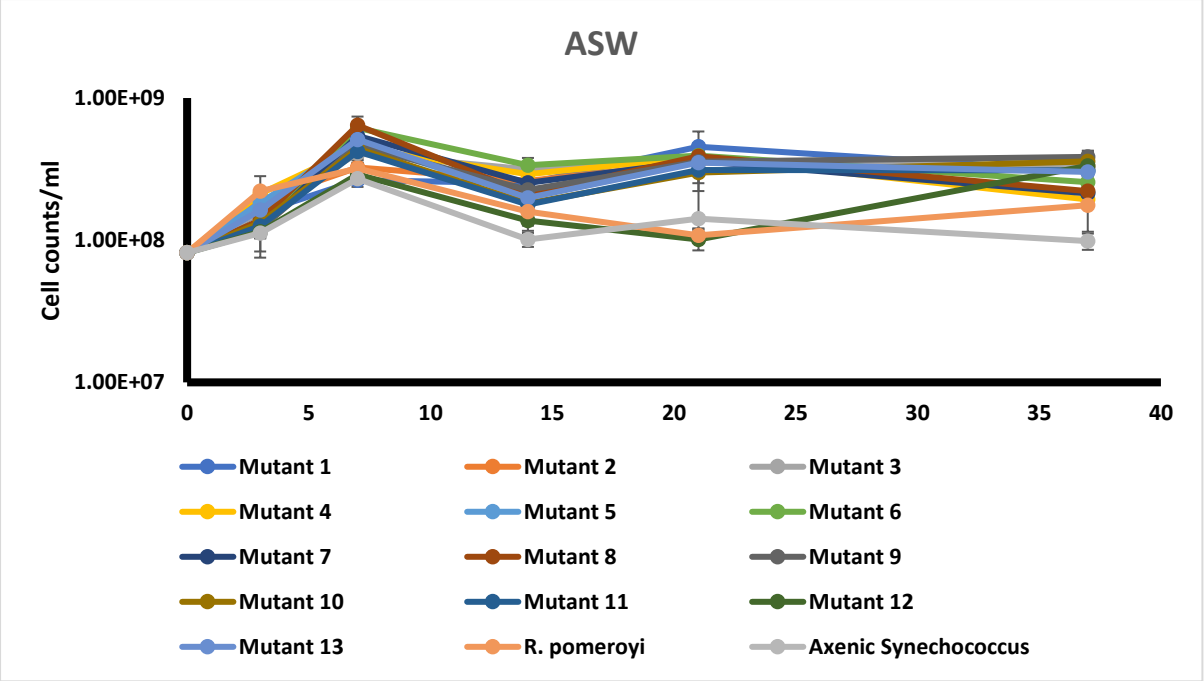

Fig. 5.2.2.3.3. Growth of *Synechococcus* WH7803 in a co-culture with different mutants of *R. pomeroyi* and wild type *R. pomeroyi* over the 37-day time course experiment in ASW medium. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

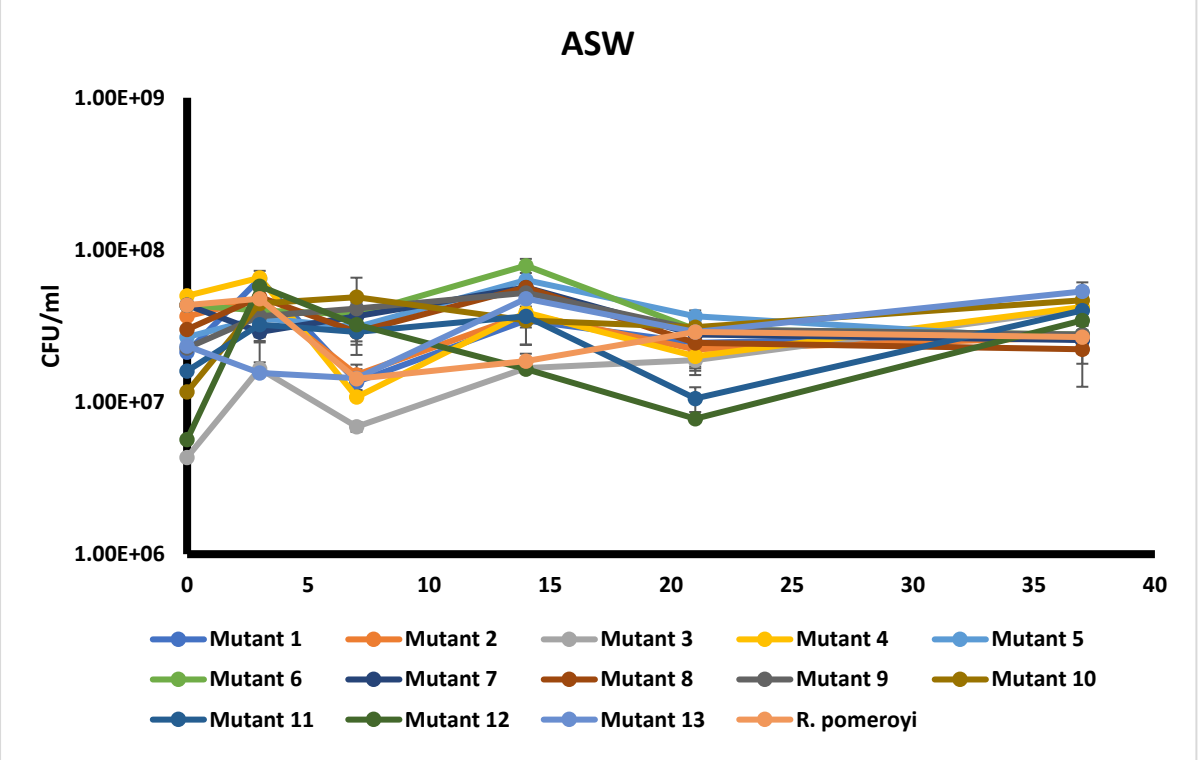

Fig. 5.2.2.3.4. Growth of different mutants of *R. pomeroyi* and wild type *R. pomeroyi* in a co-culture with *Synechococcus* WH7803 over the 37-day time course experiment in ASW medium. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

5.3 Conclusion

Marine microbes play an important role in biogeochemical cycling of carbon, nitrogen, sulfur and phosphorus. Phototrophs produce organic matter in the ocean which is used as a source of carbon and energy by heterotrophs. The latter produce exoenzymes that degrade this organic matter and provide essential nutrients to phototrophs. These exoenzymes have previously been detected in a co-culture of the model strains *R. pomeroyi* and *Synechococcus*. But very little is known about these exoenzymes because their functions were annotated only on the basis of their conserved domains. To find the substrate of these secreted proteins and to characterise them, mutants were generated that had enzymes knocked out. The mutant of pectate lyase was tested with different polysaccharides i.e. pectin, alginate, laminarin, starch and cellulose. According to the results AAV93776.1 cannot be characterised as pectate lyase because the mutant did show the same growth rate for pectin as of wild type *R. pomeroyi*. So, it could be suggested that an alternative enzyme is doing this function and that enzyme was not seen in this experiment. This growth experiment also proved that pectate hydrolase mutant was also able to grow on alginate and laminarin like wild type, but again some alternative enzyme is doing this function and that enzyme needs to be detected. So, this needs further research to find that alternative enzyme. The sialidase mutant was also tested with the monomer NANA. Wild type and sialidase mutant both should utilize this monomer, but their cell abundance was very low even compared to negative control. This means that they died because NANA was toxic to them. So, this mutant cannot be characterised as sialidase mutant. Then all the mutants were co-cultured with *Synechococcus* in natural SW and ASW for 100 days to see that if they alter the positive *Synechococcus* - *R. pomeroyi* interactions. In natural SW, it was clearly seen that growth pattern for *Synechococcus* was identical with different mutants and steady state was seen after day 21 as detected in 100- day time course experiment. Mutants 10, 12 and 13 showed detrimental effect in a co-culture with *Synechooccus* in natural SW. ASW did not show differences because it was only for 37 days. Here, there was an attempt

to characterise these exoenzymes, by testing them with different polysaccharides and co-culturing them with *Synechococcus*, but these exoenzymes needs further characterisation.

Chapter 6

Discussion

Marine microbes and their interactions are very important in marine systems, but very little is known about their interactions. Phototrophs are important as they produce oxygen molecules which we breathe and fixes CO_2. Heterotrophs regulates the biogeochemical cycles in ocean. The main aim of this project was to gain new insights on the interactions between phototrophs and heterotrophs which are essential in maintaining the nutrient balance in the oceans and figure 6.1 summarizes these interactions. To understand these interactions special attention was given to *Synechococcus* WH7803 and *R. pomeroyi* co-culture system and to the full characterization of the detected secreted proteins. The exoproteome of different model heterotrophic bacteria was also analysed in the presence of *Synechococcus*. The main focus was the detection of secreted hydrolytic enzymes.

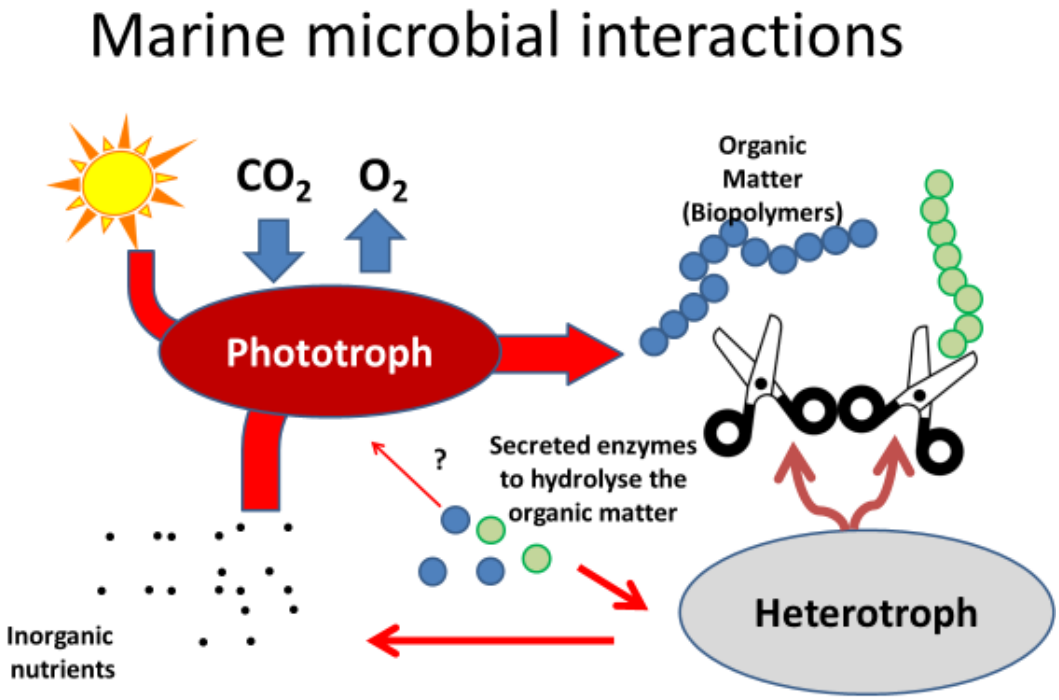

Figure 6.1: This figure summarizes the interaction between the phototrophs and the heterotrophs in which heterotrophs produces exoenzymes to degrade the DOM of phototrophs and helps in nutrient recycling.

6.1: Synechococcus-R. pomeroyi interactions: To understand the interactions between phototrophs and heterotrophs, *Synechococcus* WH7803 and *R. pomeroyi* were co-cultured for 100 days. They were co-cultured in both natural SW and ASW in which their exoproteome was analysed from different time points as mentioned in chapter 3. The different time points were analysed because single time point could give misleading results. This long–term co-culture experiment proved that their interactions are based on nutrient recycling. This has also been proved in previous experiments in which both microbes were co-cultured for over 200-days (Christie-Oleza et al., 2017). Some aspects were highlighted in this 100-day experiment which helps to understand the interactions between phototrophs and the heterotrophs, especially *Synechococcus* and *R. pomeroyi*. It has been detected previously that *R. pomeroyi* can switch between a motile and a non-motile phase depending upon the availability of nutrients and reported here by using different concentrations of organic matter (Kaur et al., 2018, Fig. 3.2.3.2.1). Also, this is in agreement with the proteomic detection of flagellin when heterotrophs usually turn off their motility during phytoplankton blooms, so that it can remain in high concentration of organic matter (Stella et al., 2008). It has been reported previously that Roseobacter strains encodes a large number of active membrane transporters which are involved in scavenging different sources of carbon and energy (Moran et al., 2004, Christie-Oleza et al., 2010). Membrane transporters plays an important role in interaction between *Synechococcus* and *R. pomeroyi* (Christie-Oleza et al., 2015). The data from chapter 3 proved that *R. pomeroyi* produces large array of specific membrane transporters to uptake amino acids, amines and carbohydrates of photosynthate produced by *Synechococcus*. So, life style of *R. pomeroyi* in this analysis revealed that it becomes motile to search for nutrients when these are scarce and to quickly uptake these nutrients it invests membrane transporters when these are abundant. Active ABC and TRAP transporters were found previously in the exoproteome of *R. pomeroyi* in a co-culture with *Synechococcus* where the total abundance of these transporters was over 40% (Christie-Oleza et al., 2015). Depletion of ROS is also an important process in marine cyanobacterium and heterotroph interactions because most of the cyanobacteria lacks catalase which helps to get rid of oxidative stress and have to depend

upon heterotroph to deplete ROS (Hunken et al., 2008; Morris et al., 2008; 2011). The data presented in chapter 3 proved that SOD was abundantly detected in the exoproteome of *Synechococcus* to deplete the ROS, even in the presence of *R. pomeroyi*. Type IV pili proteins were detected in the exoproteome of *Synechococcus* sp. WH7803 which might play an important role in cell buoyancy. To determine the role of type IV pili proteins, the best thing to do is to overexpress it in *E. coli*. Large number of hypothetical proteins were also detected which needs further characterisation because their function is unknown. This can also be done by their overexpression in *E. coli*.

6.2: Hydrolytic and interaction proteins detection in the exoproteome of R. pomeroyi:

To gain more knowledge about the nutrient recycling which are an important aspect of phototroph-heterotroph interactions, it is also important to detect and characterise the hydrolytic and interaction proteins produced by *R. pomeroyi*. An array of hydrolytic enzymes were detected overtime in the exoproteome of *R. pomeroyi* in a co-culture with *Synechococcus* to mineralise the polymeric organic matter generated by the *Synechococcus*. Some interaction related proteins i.e. RTX-related, microcystin-dependent, flagellin, GTA and PaxA were also found in the exoproteome of *R. pomeroyi*. Their functions as mentioned in table 5.2.1.1 were annotated on the basis of the conserved domain it contains, by searching for coding domain search application in NCBI. To find the substrates for hydrolytic proteins and to characterise the hydrolytic and interaction proteins, these genes were knocked out. Pectate lyase (AAV93776.1) and sialidase (AAV95476.1) were abundantly detected in time course experiment. Pectate hydrolase mutant was tested with different polysaccharides (pectin, alginate, starch, laminarin and cellulose) and sialidase mutant was tested with monomer NANA, but the results indicate that these cannot be characterised as pectate hydrolase and sialidase. Pectate hydrolase mutant and wild type *R. pomeroyi* both were able to utilise pectin which means that there is the possibility that some other alternative enzyme did this function. To find this alternative enzyme two things can be done; one is to analyse the exoproteome of pectate hydrolase mutant in the presence of all the tested substrates and second thing is to

overexpress it in *E. coli*. The reason that sialidase mutant cannot be characterised as sialidase mutant is that both wild type and sialidase mutant were died because NANA was toxic to them. Even all the mutants of hydrolytic and interaction related proteins were also co-cultured for 100 days with *Synechococcus*. In this project, there was an attempt to characterise these secreted proteins which might play an important role in interactions between *R. pomeroyi* and *Synechococcus*, but it requires more research. There were also previous attempts to characterise some exoenzymes by using fluorescently labelled compounds, but was unable to characterise them (D'Ambrosio et al., 2014, Arnosti, (2011). Definitely, to understand the marine microbial interactions, further work is required. These mutants can also be tested on ecoplates or biolog plates.

6.3: Interactions of different heterotrophs with Synechococcus: To understand the interactions between phototrophs and heterotrophs, exoproteome of 16 different heterotrophic strains was analysed in the presence/absence of *Synechococcus* WH7803 (Chapter 4). In Actinobacteria group different hydrolytic enzymes were detected such as alpha amylases, proteases, lipases and glucosidases in *S. tropica* and proteases, lipases, glucosidases and nucleases in *A. marinum*. It has been found previously that Actinobacteria group can degrade the biopolymers (Weyland et al., 1969) and this is proved in this analysis due to the detection of different hydrolytic enzymes. It was also seen that *S. tropica* produced alpha amylases in the presence of *Synechococcus*, but *Synechococcus* do not produce starch. These alpha amylases might be utilising some other polysaccharide or oligosaccharide which requires further work. This can be done by testing the mutant of alpha amylase with different polysaccharides. CFB group is also known to degrade different biopolymers such as cellulose, chitin and pectin (Reichenbach et al., 1991). The hydrolytic enzymes that were found in the exoproteome of *Polaribacter* were proteases, glucosidases, lipases, phosphatases and alpha amylase. The hydrolases in *A. machipongonensis* were proteases, glucosidases, phosphatases, sulfatases and amidases. In *G. forsetii*, the hydrolytic enzymes were abundantly detected which was about 36.47% in co-culture. It has been reported in *F.*

agariphila that it can degrade the algal polysaccharides (Mann et al., 2013). In this project, *F. agariphila* also produced different hydrolytic enzymes. The detection of an array of hydrolytic enzymes in the members of CFB group indicates that it can degrade the polysaccharides produced by *Synechococcus*. The Roseobacter clade is involved in the carbon and sulfur cycle by oxidizing the greenhouse gas carbon monoxide and produces dimethylsulfide (Geng and Belas, 2010). A clear pattern of the secreted proteins was detected in the members of Roseobacter clade in the analysis detailed in chapter 4. The presence of motility, membrane transporters and production of hydrolytic enzymes in the exoproteome of Roseobacters indicates that they play a very important role in nutrient recycling and helps to regulate the biogeochemical cycling. It is well known that Gammaproteobacteria depends upon CAZymes that can degrade the biopolymers produced by phytoplanktons (Muhlenbruch et al., 2018) and this analysis proved that it definitely produces hydrolytic enzymes to degrade the different polysaccharides. The two strains of Planctomycetes group did not grow enough in cultures, so not many proteins were detected in this group. In Verrucomicrobia, the hydrolytic enzymes were also abundantly detected especially, phosphoesterase which indicates that it interacts with *Synechococcus* by degrading its DOM. So, overall analysis of the secreted proteins in the exoproteome of 16 different heterotrophs in a co-culture with *Synechococcus* indicates that the interactions are based on nutrient recycling and they are important to understand the biogeochemical cycling. To better understand these marine microbial interactions, the time course experiment can be done as in chapter 3.

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Appendixes

Table 7.1: Protocol to prepare ASW media

Chemicals	**2.4 L**	**1 L**	**Stock**
1. $NaNO_3$	24 ml	10 ml	75 g in 1 L
2. NaCl	60 g	25 g	Use solid
3. $MgCl_2.7H_{2O}$	24 ml	10 ml	200 g in 1 L
4. KCl &$CaCl_2.2H_2O$	12 ml	5 ml	50 g in 500 ml of each
5. $MgSO_4$	24 ml	10 ml	175 g in 500 ml
6. Trizma	13.2 ml	5.5 ml	100 g in 500 ml
7. $K_2H_3PO_4$	6 ml	2.5 ml	6 g in 500 ml
8. Trace metals	2.4 ml	1 ml	

7.1.1 Weigh 60 g of NaCl (for 2.4 L of media) and add milliQ water in it.

7.1.2 Do caliberation. Clean the electrode with milliQ water. Put the electrode in buffer 4 for some time and press standardise for twice and it will show the PH. Again, clean the electrode with milliQ and repeat it for buffer 7 and 10.

7.1.3 Pipette out the rest of chemicals.

7.1.4 Adjust PH to 8 by adding concentrated HCl.

7.1.5 Autoclave the media for 20 min at 121℃.

Table 7.2: Protocol to prepare MMM media plates for conjugation

Chemicals	**100 ml**	**Stock**
1. Sea salts	3 g	
2. Bacto-agar	1.5 g	
3. HEPES	1 ml	11.915 g in 50 ml (adjust PH 8 with 4-5 pellets of NaOH, filter sterile)
4. Na2HPO4	0.5 ml	1.41 g in 50 ml (filter sterile)
5. FeCl3	1 µl	0.675 g in 50 ml (filter sterile)
6. Vitamins	100 µl	
7. MMA	300 µl	2.876 g in 50 ml (filter sterile)
8. Succinate	2 ml	12.5 g in 50 ml (filter sterile)

7.2.1 Weigh 3 g of sea salts and add 100 ml of milliQ water in it.

7.2.2. Then add 1.5 g of Bacto-agar, mix well and autoclave for 20 min at 121℃.

7.2.3 Cool down the media and add rest of chemicals including antibiotic (100 µl) of interest for the mutation.

7.2.4. Prepare the plates and dry them properly.

Table 7.3: Protocol to prepare ASW Pom media

Chemicals	**1 L**	**Stock**
1. NaCl	25 g	Use solid
2. $MgCl_2.7H_{2O}$	10 ml	200 g in 1 L
3. KCl &$CaCl_2.2H_2O$	5 ml	50 g in 500 ml of each
4. $MgSO_4$	10 ml	175 g in 500 ml
5. Trizma	5.5 ml	100 g in 500 ml
6. $K_2H_3PO_4$	2.5 ml	6 g in 500 ml
7. Trace metals	1 ml	
8. Yeast extract	10 ml	5 g in 1L
9. $(NH_4)_2\ SO_4$	5 ml	0.5 M
10. Succinate	20 ml	25 g in 1 L
11. Vitamins	2 ml	

7.3.1 Weigh 25 g of NaCl (for 1 L of media) and add milliQ water in it.

7.3.2 Do caliberation. Clean the electrode with milliQ water. Put the electrode in buffer 4 for some time and press standardise for twice and it will show the PH. Again, clean the electrode with milliQ and repeat it for buffer 7 and 10.

7.3.3 Pipette out the rest of chemicals except succinate and vitamins.

7.3.4 Adjust PH to 8 by adding concentrated HCl.

7.3.5 Autoclave the media for 20 min at 121℃ and then add succinate and vitamins (filter sterilised) when the media gets cooled down.

Table 7.4: Evolution of the concentration (in cell/ml) of *Synechococcus* during the period of incubation i.e. between times T0 (inoculation) and T3 (end of 3 days of incubation). (Modified from MSc student Natacha Chenevoy report).

Name of the strain in co-culture with *Synechococcus*	**Concentration at T0**	**Concentration at T3**
Salinispora tropica	$6.4 \cdot 10^6$	$11 \cdot 10^6$
Aeromicrobium marinum	$6.4 \cdot 10^6$	$18 \cdot 10^6$
Polaribacter sp.	$6.4 \cdot 10^6$	$17 \cdot 10^6$
A. machipongonensis	$3,2 \cdot 10^6$	$11 \cdot 10^6$
Gramella forsetii	$6.4 \cdot 10^6$	$17 \cdot 10^6$
Formosa agariphila	$3,2 \cdot 10^6$	$10,5 \cdot 10^6$
Ruegeria pomeroyi	$3,2 \cdot 10^6$	$11 \cdot 10^6$
Roseobacter denitrificans	$3,2 \cdot 10^6$	$11 \cdot 10^6$
Dinoreseobacter shibae	$3,2 \cdot 10^6$	$11 \cdot 10^6$
Pseudoalteromonas citrea	$3,2 \cdot 10^6$	$10,4 \cdot 10^6$
Alteromonas macleodii	$3,2 \cdot 10^6$	$9,0 \cdot 10^6$
Marinobacter adhaerens	$3,2 \cdot 10^6$	-
Pseudomonas stutzeri	$3,2 \cdot 10^6$	$9,5 \cdot 10^6$
Planctomyces limnophilus	$6.4 \cdot 10^6$	$22 \cdot 10^6$
Rhodopirellula baltica	$3,2 \cdot 10^6$	$7,0 \cdot 10^6$
Verrucomicrobiae bacterium	$3,2 \cdot 10^6$	$11 \cdot 10^6$

Table 7.5: Comparison of cell concentration (in cell/ml) at T0 (inoculation) and T3 (end of the 3 days of incubation) for each heterotrophic strain in mono-culture and co-culture. (Modified from MSc student Natacha Chenevoy report).

Heterotrophs	**Concentration at T0**		**Concentration at T3**	
	Mono-culture	**Co-culture**	**Mono-culture**	**Co-culture**
Salinispora tropica	$3,0 \cdot 10^5$	$3,0 \cdot 10^5$	NA (clamps)	NA (clamps)
Aeromicrobium marinum	$7,8 \cdot 10^7$	$7,8 \cdot 10^7$	$8,0 \cdot 10^7$	$1,2 \cdot 10^8$
Polaribacter sp.	$7,8 \cdot 10^7$	$7,8 \cdot 10^7$	$3,0 \cdot 10^7$	$6,0 \cdot 10^7$
A. machipongonensis	$4,8 \cdot 10^7$	$4,8 \cdot 10^7$	$5,0 \cdot 10^8$	$1,0 \cdot 10^8$
Gramella forsetii	$1,3 \cdot 10^8$	$1,3 \cdot 10^8$	$2,0 \cdot 10^7$	$1,0 \cdot 10^7$
Formosa agariphila	$4,8 \cdot 10^6$	$4,8 \cdot 10^6$	$2,0 \cdot 10^6$	$2,0 \cdot 10^6$
Ruegeria pomeroyi	$1,2 \cdot 10^7$	$1,2 \cdot 10^7$	$5,0 \cdot 10^8$	$7,0 \cdot 10^7$
Roseobacter denitrificans	$1,0 \cdot 10^8$	$1,0 \cdot 10^8$	$2,0 \cdot 10^8$	$2,0 \cdot 10^8$
Dinoreseobacter shibae	$9,0 \cdot 10^7$	$9,0 \cdot 10^7$	$1,2 \cdot 10^9$	$2,0 \cdot 10^8$
Pseudoalteromonas citrea	$5,4 \cdot 10^5$	$5,4 \cdot 10^5$	$2,0 \cdot 10^7$	$6,0 \cdot 10^6$
Alteromonas macleodii	$1,8 \cdot 10^7$	$1,8 \cdot 10^7$	$3,0 \cdot 10^8$	$3,0 \cdot 10^7$
Marinobacter adhaerens	$5,4 \cdot 10^6$	$5,4 \cdot 10^6$	$5,0 \cdot 10^8$	$3,0 \cdot 10^7$
Pseudomonas stutzeri	$7,8 \cdot 10^7$	$7,8 \cdot 10^7$	$6,0 \cdot 10^8$	$9,0 \cdot 10^7$
Planctomyces limnophilus	$1,8 \cdot 10^7$	$1,8 \cdot 10^7$	NA (death of culture	NA (death of culture
Rhodopirellula baltica	$1,8 \cdot 10^7$	$1,8 \cdot 10^7$	$8,0 \cdot 10^6$	$6,0 \cdot 10^6$
Verrucomicrobiae bacterium	$6,0 \cdot 10^7$	$6,0 \cdot 10^7$	$4,0 \cdot 10^7$	$5,0 \cdot 10^7$

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100 Days of marine *Synechococcus–Ruegeria pomeroyi* interaction: A detailed analysis of the exoproteome

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Summary

Marine phototroph and heterotroph interactions are vital in maintaining the nutrient balance in the oceans as essential nutrients need to be rapidly cycled before sinking to aphotic layers. The aim of this study was to highlight the molecular mechanisms that drive these interactions. For this, we generated a detailed exoproteomic time-course analysis of a 100-day co-culture between the model marine picocyanobacterium *Synechococcus* sp. WH7803 and the *Roseobacter* strain *Ruegeria pomeroyi* DSS-3, both in nutrient-enriched and natural oligotrophic seawater. The proteomic data showed a transition between the initial growth phase and stable-state phase that, in the case of the heterotroph, was caused by a switch in motility attributed to organic matter availability. The phototroph adapted to seawater oligotrophy by reducing its selective leakiness, increasing the acquisition of essential nutrients and secreting conserved proteins of unknown function. We also report a surprisingly high abundance of extracellular superoxide dismutase produced by *Synechococcus* and a dynamic secretion of potential hydrolytic enzyme candidates used by the heterotroph to cleave organic groups and hydrolase polymeric organic matter produced by the cyanobacterium. The time course dataset we present here will become a reference for understanding the molecular processes underpinning marine phototroph-heterotroph interactions.

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Introduction

The ocean is the Earth's largest biome covering 70% of the world's surface. Marine systems play a major role in global climate regulation, not only due to their ability to store and transport heat, but also because of the constant atmosphere—ocean exchange of CO_2. Oceans are major carbon reservoirs and are known to buffer anthropogenic carbon emissions by drawing CO_2 from the atmosphere and burying it in the deep ocean, a process known as the biological carbon pump (Jiao and Zheng, 2011; De La Rocha and Passow, 2014). This is a process whereby CO_2 in the upper ocean is fixed by photosynthetic primary producers to form organic matter that is then transported to the deeper ocean by sedimenting particulate organic matter (POM) and the drawdown of dissolved organic matter (DOM) through mixing and dwelling (Jiao *et al.*, 2010). Marine microbes are also major drivers of other global biogeochemical cycles such as those of nitrogen and sulphur (Karl and Church, 2014).

Marine picocyanobacteria, mainly belonging to the genera *Prochlorococcus* and *Synechococcus*, are numerically the world's dominant photosynthetic primary producers and a major component of marine phytoplankton. Despite being outnumbered by the heterotrophic community, these cyanobacteria are responsible for half of the marine primary production and play a key role in sustaining marine food webs (Falkowski, 2012). Hence, phytoplankton are at the base of the marine food chain feeding the ecosystem with DOM and POM that is released via cell lysis (e.g., cell death, inefficient grazing and viral lysis) or other cellular processes (e.g., outer membrane vesicles, active efflux processes or permeable membrane leakage) (Biller *et al.*, 2014; Christie-Oleza *et al.*, 2015a,; Grossowicz *et al.*, 2017). Most of this organic matter will be used by the heterotrophic bacterioplankton as their main source of carbon and energy, returning inorganic nutrients to the phototrophic community (Christie-Oleza *et al.*, 2017). The *Roseobacter* group of the class Alphaproteobacteria is an important component of the microbial community within the marine euphotic layer, accounting for up to 20% of the sea surface bacterioplankton (Wagner-Dobler and Biebl, 2006). This group of heterotrophs generally shows a

positive correlation with the phytoplankton community and forms intimate associations with specific phytoplankton groups (Giebel *et al*., 2011; Morris *et al*., 2012). Interestingly, marine *Roseobacter* strains present a large genomic capability and metabolic versatility to use an array of organic substrates found within phytoplankton exudates and, hence, they are one of the first bacterioplankton groups to react to the input of organic matter produced, for example, during phytoplankton blooms (Newton *et al*., 2010; Romera-Castillo *et al*., 2011; Christie-Oleza *et al*., 2012a; Buchan *et al*., 2014; Landa *et al*., 2016; Simon *et al*., 2017). The strain *Ruegeria pomeroyi* DSS-3 was the first roseobacterium to have its genome sequenced (Moran *et al*., 2004) and has served as a model organism to study biogeochemical, ecological and physiological strategies of this group of heterotrophic marine bacteria (Christie-Oleza and Armengaud, 2015).

Around 70% of the DOM in the oceans is considered of low-molecular weight ($<$ 1 kDa) (Benner, 2002). The other 30% of DOM is of high molecular weight ($>$ 1 kDa) and, curiously, is much less refractory and, hence, more readily degraded than the low-molecular weight fraction (Decho and Gutierrez, 2017). Most of the high molecular weight DOM is in the form of biopolymers, and because biological membrane systems are only permeable to molecules smaller than 0.6 kDa (Weiss *et al*., 1991), exoenzymes or ectoenzymes play a key pivotal role in polymeric DOM hydrolysis and assimilation (Vetter and Deming, 1999) as these biopolymers must be hydrolyzed outside the cell before they can be taken up by most organisms (i.e., non-grazing organisms). The activities of secreted enzymes in marine microbes has generally been assessed through the use of fluorescently labeled substrates (Karner and Herndl, 1992; Martinez *et al*., 1996; D'Ambrosio *et al*., 2014; Arnosti, 2015), simple observation of bacterial growth on different polymeric substrates (e.g., Mitulla *et al*., 2016) or, more rarely, identifying and characterizing the actual enzymes involved in hydrolyzing the DOM (e.g., Hehemann *et al*., 2014; Xing *et al*., 2015). Interestingly, marine bacterial exoenzymes are proving highly distinct from their well-characterized terrestrial counterparts (Michel and Czjzek, 2013) and are currently poorly identified.

The use of shotgun proteomics has become a powerful tool for detecting the array of proteins present in the extracellular medium of an organism under different experimental conditions (Armengaud *et al*., 2012) and, hence, a reliable high throughput method for identifying key secreted enzymes and proteins involved in cell-to-cell and cell–environment interactions. We previously analyzed the exoproteome of various *Roseobacter* strains grown in rich media and showed a diversity of trophic strategies within this clade, that is, through the abundant detection of secreted nutrient transporters, mobility proteins, adhesion-like proteins or toxins (Christie-Oleza *et al*., 2012b). Nevertheless, *Roseobacter* strains induced a completely different array of secreted proteins when grown in the presence of a more realistic source of organic matter, that is, DOM and POM produced by marine *Synechococcus*, with an increase in proteins involved in motility, microbial interactions, hydrolytic activities and capsid proteins of the genetic transfer agent (GTA) encoded in the genome and a strong decrease in the secretion of toxin-like proteins (Christie-Oleza *et al*., 2015b).

Synechococcus species generate large amounts of organic matter and requires the presence of a specialized heterotrophic community to remineralise the leaked photosynthate and obtain a constant feed-back of inorganic nutrients (Christie-Oleza *et al*., 2017). Based on this basic principle of phototroph–heterotroph interaction, illuminated *R. pomeroyi–Synechococcus* sp. co-cultures are able to survive for extended time periods both in mineral enriched media and natural oligotrophic seawater (Christie-Oleza *et al*., 2017). In this study, we analyzed for the first time the exoproteome of *R. pomeroyi–Synechococcus* co-cultures under an extended time period (i.e., 100 days) both in nutrient-enriched and natural oligotrophic seawater in order to generate a unique time course dataset that would highlight the molecular mechanisms involved in this dependent microbial interaction over time. We therefore aimed to (i) determine protein pattern shifts over the 100-day time course and observe whether culture stability is reflected by a consistent exoproteome; (ii) identify culture stages and frame the trophic strategy of each microbe at each time point; and (iii) identify the secreted hydrolytic enzymes produced by the heterotroph and evaluate their variations over time.

Results and discussion

Correlation between culture growth and exoproteomes

In both nutrient-rich and oligotrophic media, the sustained survival of the co-culture comes as a consequence of nutrient cycling between the phototroph and heterotroph (Christie-Oleza *et al*., 2017). Here, the growth of *Synechococcus* sp. WH7803 and *R. pomeroyi* DSS-3 during the 100 days co-culture in both natural oligotrophic seawater (SW) and enriched artificial seawater (ASW) (Fig. 1) showed the expected trends as previously reported (Christie-Oleza *et al*., 2017). While *Synechococcus* sp. WH7803 reaches high cell densities in ASW ($>$ 10^9 cells ml^{-1}) and growth is limited by light, in natural SW the cyanobacterium is limited by the availability of inorganic nutrients and cell densities reached 10^5 cells ml^{-1}, numbers that are similar to those observed in natural marine ecosystems (Parsons *et al*., 2012). The heterotroph, *R. pomeroyi* DSS-3, is limited by the availability of organic carbon and vitamins regenerating essential nutrients for the phototroph. *Synechococcus–R. pomeroyi* co-cultures can persist over

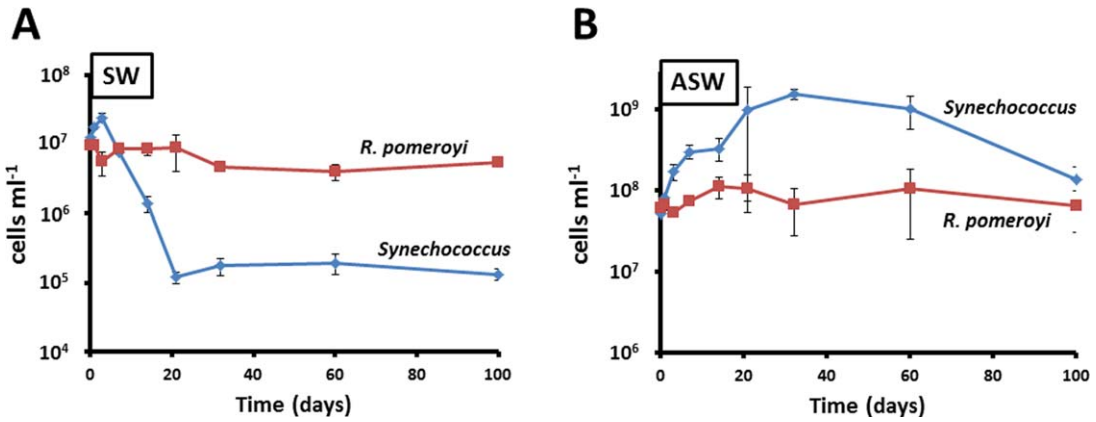

Fig. 1. Growth curves of *R. pomeroyi* DSS-3 and *Synechococcus* WH7803 over the 100-day time course experiment in natural SW (A) and ASW medium (B). The average value of triplicate cultures ($n = 3$) is shown in panels (error bars show standard deviation).

200 days (Christie-Oleza *et al.*, 2017), but a sTable state is reached after just 21 days in natural SW (Fig. 1). However, in rich ASW medium, *Synechococcus* sp. WH7803 only enters the sTable state after 100 days when cell densities drop 10-fold and stabilise at 10^8 cells ml^{-1} (Christie-Oleza *et al.*, 2017).

Exoproteomes tend to reflect microbial adaptive strategies (Christie-Oleza *et al.*, 2012bb), and as expected, the variations observed in the exoproteomes presented here corresponded nicely with the different growth-phase physiologies observed in SW and ASW grown co-cultures as shown by the principal component analyses (PCA) using the normalized exoproteomic data obtained from *Synechococcus* sp. WH7803 and *R. pomeroyi* over the time course experiment. The sum of the two first principal components represented 82% and 93% of the variability within the exoproteome of *Synechococcus* sp. WH7803 and *R. pomeroyi* over the 100 days, respectively (Fig. 2).

PCA of the exoproteome of Synechococcus *sp. WH7803.* In natural SW, the exoproteome of *Synechococcus* showed a distinct shift between time points 1–14 and 21–100 days incubation (Fig. 2A), which coincides with the co-culture entering the sTable state equilibrium observed at day 21 (Fig. 1). Interestingly, the exoproteome of ASW-grown *Synechococcus* sp. WH7803 remained similar over the 100 days experiment, except for time point 21 which marks the transition from the exponential to the sTable state phase (Fig. 1). Curiously, time point 21 grouped with the sTable state time points of *Synechococcus* grown in oligotrophic SW (i.e., 21–100 days; Fig. 2A), suggesting a transitory peak of starvation in ASW media.

PCA of the exoproteome of R. pomeroyi. The heterotroph showed a similar transition over time in both nutrient-poor and -rich media (Fig. 2B), mainly marked by the shift from exponential and sTable state cultures. Nevertheless,

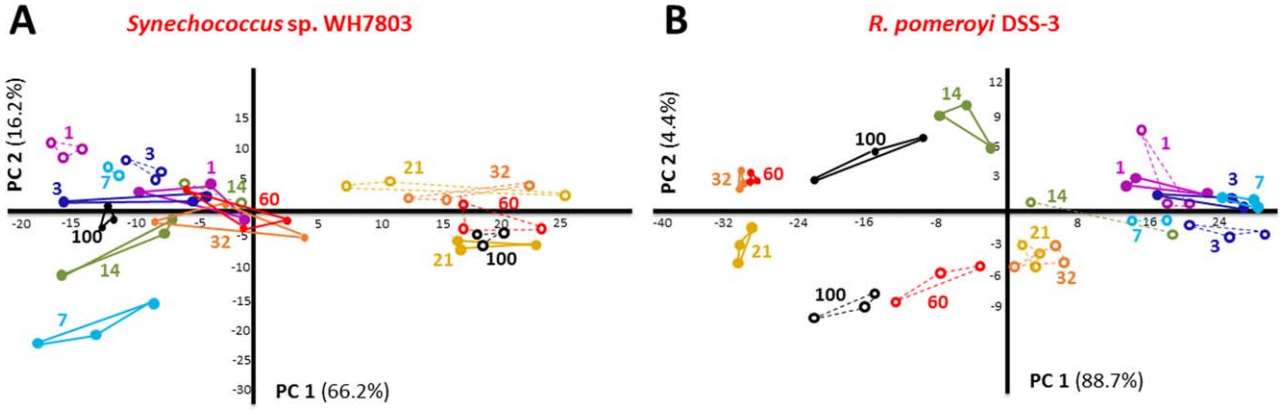

Fig. 2. PCA of the normalized exoproteomes of *Synechococcus* sp. WH7803 (A) and *R. pomeroyi* DSS-3 (B) when grown in co-culture in ASW medium (solid circles and lines) and natural SW (open circles and dashed lines). Numbers refer to the culture day that the samples were collected.

the slight divergence between SW and ASW exoproteomes was mainly resolved by the principal component 2 (Fig. 2B), suggesting a distinctness between nutrient-rich and oligotrophic culture conditions.

Although the transitions that were observed over time are likely caused by the acclimation of the microbial populations to the varying conditions of the culture, genetic modifications (as demonstrated in Zambrano *et al.*, 1993) cannot be ruled out and, hence, further research is needed to determine if genetic evolution also occurs during these long-term co-cultures.

The exoproteome of Synechococcus *over the 100-day time course*

Just 187 and 221 of the 681 polypeptides detected in the co-culture's exoproteome belonging to *Synechococcus* already represented 95% of the total protein abundance in SW and ASW, respectively. These proteins were grouped into functional categories (Supporting Information Table S4) and represented in Figure 3A.

Photosynthesis and basic cellular processes. Synechococcus sp. release considerable amounts of organic matter not only in the form of carbohydrates (Biersmith and Benner, 1998; Bertlisson *et al.*, 2005) but also in the form of protein (Christie-Oleza *et al.*, 2015a; 2017). Here, proteins involved in photosynthesis are among the most abundant categories found in the exoproteome of *Synechococcus* sp. WH7803, representing around 32% of the exoproteome (Table 1). Interestingly, this percentage is strikingly similar to the amount of photosynthetic proteins observed in cellular proteomic analyses (Table 1; Christie-Oleza *et al.*, 2017). While this may suggest that the exoproteome of *Synechococcus* is made entirely of proteins generated from cell lysis, other indicators such as the amount of cytoplasmic or ribosomal proteins are not in agreement. While ribosomal proteins in *Synechococcus* cells represent between 5.5% and 6.5% of the total cellular proteome, only between 1.0% and 1.6% of relative abundance of these proteins are found in the extracellular fraction (Table 1), suggesting that between 18% and 25% of the exoproteome may well come from cell lysis. When using cytoplasmic proteins as an indicator, the average percentage of predicted cell lysis increases to 50% (Table 1), but with bursts that coincide with the cell lysis expected from the growth curves. For example, the drastic reduction of *Synechococcus* cell abundance observed at days 7 and 14 under SW conditions (Fig. 1A) coincides with an increase in detection of cytoplasmic proteins in the exoproteome (i.e., 25–30%), suggesting that, during these time points, between 65% and 77% of the extracellular proteins may come from cell lysis. Nevertheless, at other time points cell lysis remains stably between 40% and 50%. It is interesting to note that some of these predicted

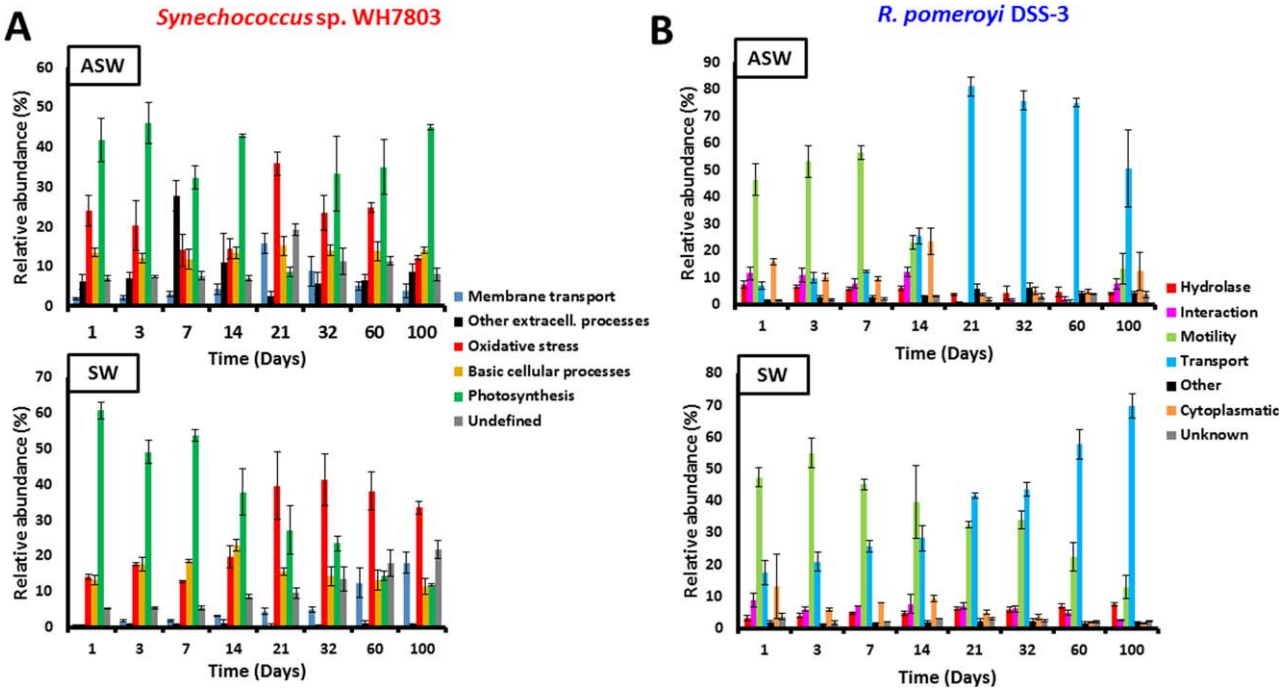

Fig. 3. Functional category abundance of protein found in the exoproteomes of *Synechococcus* sp. WH7803 (A) and *R. pomeroyi* DSS-3 (B) when grown in co-culture in ASW medium and natural SW. The average value of triplicate cultures analyses (n = 3) are shown (error bars show standard deviation).

Table 1. Relative abundance of ribosomal proteins and proteins form the photosynthetic apparatus detected in *Synechococcus* sp. WH7803 proteome datasets.

	Cellular fraction[a]		Exoproteome fraction[b]			
	SW	ASW	SW		ASW	
Synechococcus						
Cytoplasmic proteins[c]	38.5 ± 1.1	33.1 ± 1.0	19.4 ± 4.9	50%	16.6 ± 2.1	50%
Ribosomal proteins	6.5 ± 0.9	5.5 ± 1.0	1.6 ± 0.8	25%	1.0 ± 0.6	18%
Photosynthetic apparatus	35.9 ± 1.3	39.4 ± 1.8	31.1 ± 19.8	87%	32.2 ± 11.6	82%
Phycobilisomes	29.8 ± 1.1	28.4 ± 1.6	28.2 ± 19.2	95%	29.2 ± 11.7	103%
Other elements	6.1 ± 0.4	11.1 ± 0.3	2.9 ± 0.8	48%	2.9 ± 0.7	26%
R. pomeroyi						
Cytoplasmic proteins[c]	57.6 ± 1.3	n.a.	6.0 ± 2.5	10%	10.4 ± 6.4	18%
Ribosomal proteins	8.0 ± 0.4	n.a.	0.2 ± 0.2	3%	0.8 ± 0.7	10%

n.a., not applicable due to the low number of proteins detected for *R. pomeroyi* in this analysis.
a. Data obtained from cellular proteomes published in Christie-Oleza and colleagues (2017). Standard deviation from triplicate samples is shown.
b. This study. Standard deviation from triplicate experiments from all eight sampled time points is shown. Percentages were calculated by dividing the protein relative abundance obtained from exoproteome analyses by those obtained in cellular fractions.
c. Cytoplasmic proteins were predicted using the prediction server PSORTb.

cytoplasmic proteins are more abundant in the exoproteome than in the cellular fraction suggesting one of the following three possibilities: (i) they are more sTable in the extracellular milieu than other cytoplasmic proteins and, therefore, over time they are enriched in the exoproteome; (ii) they might actually be actively secreted; and (iii) they are 'selectively leaked' as suggested by others (Grossowicz *et al*., 2017). One of these proteins is the nucleotide-binding protein SynWH7803_1823, a protein predicted to be involved in the temporal control of bacteriophage gene transcription, which represents 2.4% of *Synechococcus* exoproteome (almost 12% of the predicted cytoplasmic proteins in this fraction), whereas it only represents 0.1% in the cellular proteome. Proteins involved in photosynthesis are another example of unequal accumulation in the extracellular milieu or 'selective leakiness', and different elements of the photosynthetic apparatus are differentially partitioned, such as those from the phycobilisome (Table 1). Phycobilisomes should be localized on the cytoplasmic side of the thylakoidal membrane, but they seem to be enriched in the exoproteome of *Synechococcus*. Interestingly, the presence of these proteins from the photosynthetic antenna shows a strong decrease in the natural SW exoproteome over time (from 55% to 5% of the exoproteome; Fig. 3A and Supporting Information Table S4), suggesting a reduction in leakiness or a decrease in the production of cellular phycobilisomes as a consequence of nutrient stress. Protein trafficking in cyanobacteria remains poorly characterized (Schneider, 2014) and the 'selective leakage' observed here, such as of predicted cytoplasmic proteins or phycobilisomes, requires further research.

Membrane transport. The periplasmic-binding component of ABC transporters is commonly found in microbial exoproteomes (Christie-Oleza and Armengaud, 2010; Johnson-Rollings *et al*., 2014) and it has been suggested that some of these proteins could be intentionally translocated from the periplasm to the extracellular space (Giner-Lamia *et al*., 2016). The detection of membrane transporters is a good indicator of (i) the nutrients that are targeted by each organism in a community (Christie-Oleza *et al*., 2017) and (ii) an organisms' nutrient stress within the system (Saito *et al*., 2014). As expected, *Synechococcus* sp. WH7803 predominantly targets inorganic nutrients, mainly phosphate and metals such as iron (Supporting Information Table S4). Up to three different periplasmic substrate-binding proteins for phosphate were detected in the ASW condition, although only one of them (i.e., SynWH7803_1045) was abundantly detected in both enriched and oligotrophic conditions. Interestingly, none of these periplasmic-binding proteins co-localizes in the genome with the other components of the ABC transporter for phosphate (i.e., permease and ATPase). *Synechococcus* shows a clear rise in P starvation over time under oligotrophic SW conditions as seen by the progressive increase in abundance of the periplasmic substrate binding protein for phosphate SynWH7803_1045 from 0.7% at day 1 to over 14% after the 100 day incubation (Supporting Information Table S4). A similar trend is observed for porins that are linked to P stress (SynWH7803_0993, 0.1–3.1%; SynWH7803_2236, below 0.1–0.7%) and an outer membrane efflux protein involved in protein secretion which needs further characterization (SynWH7803_2199, below 0.01–0.11%). In nutrient-enriched ASW media, the cyanobacterium has a peak in all nutrient transport proteins (i.e., P and metals) at day 21, which then drops until day 100 (Fig. 3A), an trend that fits the variability observed in the PCA plot in Figure 2A.

Oxidative stress. Apart from nutrient recycling (Christie-Oleza *et al*., 2017), the scavenging of reactive oxygen species (ROS) is considered a pivotal process in marine cyanobacteria – heterotroph interactions where the cyanobacterium, mostly lacking of catalase, relies on the heterotroph for depleting ROS (Hunken *et al*., 2008; Morris *et al*., 2008; 2011). Nevertheless, while catalase only catalyzes the decomposition of hydrogen peroxide, it is the enzyme superoxide dismutase (SOD) which helps deal with superoxide (O^{2-}). SODs are found in all marine cyanobacteria although with different metal cofactors (Scanlan *et al*., 2009). While Ni-containing SODs are prevalent in catalase-lacking strains, Fe-containing SODs prevail in catalase encoding cyanobacteria. Cellular proteomes of *Synechococcus* sp. WH7803 generally show below 1% abundance of this enzyme (Christie-Oleza *et al*., 2017), but SOD has also been detected in the exoproteomes of different marine *Synechococcus* strains ranging between 0.7% and 3.4% (Christie-Oleza *et al*., 2015a). SODs are possibly periplasmic-located enzymes with a non-canonical secretion system, but may also be 'unavoidably leaked' through the outer membrane, reinforcing the idea that ROS scavenging can be a public goods process as suggested by others (Morris *et al*., 2012). In this study, the abundance of the Fe-containing SOD in the exoproteome of *Synechococcus* sp. WH7803 was surprisingly high throughout the entire time course experiment both in SW and ASW averaging 24% and 19% of total protein abundance, respectively (Supporting Information Table S4), and highlighting the relevance of this enzyme for dealing with ROS. The fact it was detected in both nutrient conditions is an indicator that its abundance is not an artefactual result and its variable abundance in ASW suggests it does not come as a consequence of its accumulation in the milieu. The second SOD encoded by *Synechococcus* sp. WH7803, the copper-contain protein SynWH7803_0951, was also detected in both SW and ASW exoproteomes with an average detection of 0.19% and 0.03%, respectively.

Other extracellular processes. The variable abundance of structural type IV pili proteins in the exoproteome of *Synechococcus* sp. WH7803 is the most remarkable aspect within this functional category (Supporting Information Table S4). In a previous study, we already reported a high abundance of the pili proteins SynWH7803_1795 and SynWH7803_1796 (17.9% and 1.3%, respectively), which was exclusive to this strain (Christie-Oleza *et al*., 2015a). The time course experiment has allowed us to assign the production of these proteins to nutrient-rich conditions with a peak of SynWH7803_1795 detection at day 7 (25.7%) and an average detection of 8.1% over the 100 days, whereas the detection was low ($< 0.1\%$) in natural SW conditions. The remarkable ecological and physiological roles of the pili need further characterization although a recent study in *Synechococcus elongatus* suggests this structure may be involved in cell buoyancy (Nagar *et al*., 2017). Interestingly, both alkaline phosphatases were detected in the exoproteome of *Synechococcus* sp. WH7803 but only in low abundance and peaked during day 21 in ASW (Supporting Information Table S4).

Secreted proteins of unknown function. Proteins of unknown function tend to predominate in the secreted fraction, highlighting the poor knowledge we currently have on microbe–environment interactions (Christie-Oleza *et al*., 2015a). Datasets such as the one we present here are useful for flagging proteins of unknown function that are abundant and, hence, may have an important role and also to shed light on their possible function by monitoring their abundance over time. Interestingly, the five most abundant proteins in this category were common in both SW and ASW conditions (Supporting Information Table S4), but these showed interesting shifts in abundance over time (Fig. 4). The conserved proteins in bacteria or marine cyanobacteria SynWH7803_0982, SynWH7803_2169 and SynWH7803_1017 showed very similar behavior, with a progressive increase in oligotrophic SW and a peak in abundance at day 21 in ASW (Fig. 4), a time point when *Synechococcus* sp. WH7803 was nutrient-starved as highlighted above. Hence, these proteins may have a role in response to nutrient stress. In contrast, protein SynWH7803_1824, which is unique to strain WH7803 and conserved only among *Burkholderia*, remained at a constant low abundance but became highly abundant in SW over time (Fig. 4). Finally, protein SynWH7803_1556 showed identical behavior in both culture conditions over time but with a lag of a few days in ASW.

The exoproteome of R. pomeroyi *over time*

From the 585 proteins detected in the exoproteome of the co-culture belonging to *R. pomeroyi*, 184 and 222 represented over 95% of the total abundance in SW and ASW, respectively, and were grouped into functional categories (Supporting Information Table S5) and represented in Figure 3B.

Cytoplasmic proteins. As mentioned earlier, the detection of predicted cytoplasmic proteins and ribosomal proteins in the exoproteomes can be used as indicators of cell lysis. While the abundance of ribosomal proteins in cellular fractions of *R. pomeroyi* usually represents over 8% of the total proteome (Table 1), here we observed only 0.2% and 0.8% of these proteins in the exoproteomes of SW and ASW co-cultures, respectively, suggesting that between 3% and 10% of the proteins detected in this study may come from cell lysis. Nevertheless, a higher cell lysis is suggested when predicted cytoplasmic proteins is used as an indicator (i.e., 10–18%; Table 1). There are several

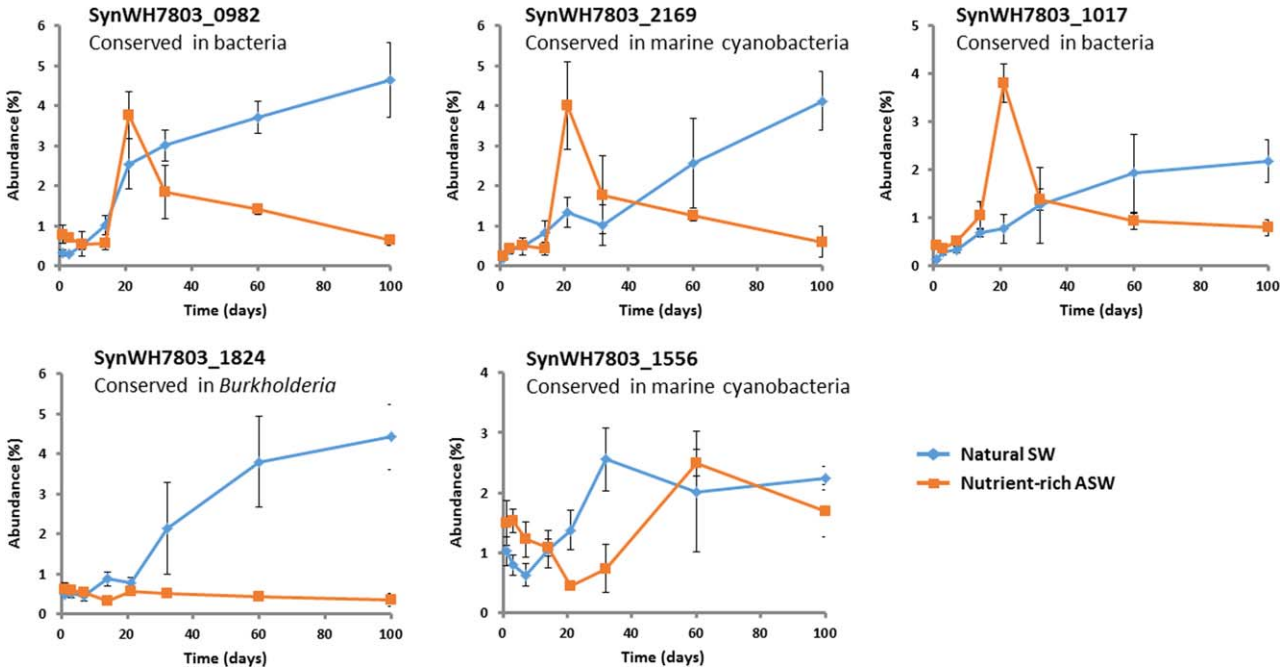

Fig. 4. Variation of the five most abundant hypothetical proteins in the exoproteome of *Synechococcus* sp. WH7803 over time. The average value of triplicate cultures analyses ($n = 3$) are shown (error bars show standard deviation).

reasons that could explain this discrepancy: (i) that dying cells reduce their ribosomal protein content; (ii) the size of ribosomal complexes makes them less 'leaky' than other smaller cytoplasmic proteins; and (iii) the prediction of protein trafficking or selective leakiness has been understudied. For example, under both conditions (i.e., SW and ASW), the five most abundant cytoplasmic proteins in the exoproteome of *R. pomeroyi* already contributed to over 50% of the cytoplasmic category, being three of them common between both conditions (i.e., isocitrate dehydrogenase, a hypothetical protein with high similarity to a phosphoenolpyruvate mutase and imidazoleglycerol-phosphate dehydratase; Supporting Information Table S5), hinting that these proteins, either moonlight (Jeffery, 2009), are dually secreted at least to the periplasm, or are highly sTable in the extracellular milieu and accumulate over time.

Motility. Ruegeria pomeroyi is a flagellum-propelled motile bacterium. The extracellular flagellin filament and hook are rarely found in the cellular proteome as they are easily sheared off during sample processing and remain in the exoproteomic fraction. The flagellin was detected in very high abundance in the exoproteomes of both SW and ASW conditions (35% and 23%, respectively). During initial culture stages flagellin represented over 50% of the total protein abundance, but while its detection only reduced progressively over time in natural SW (down to 13% at day 100), it dropped to under 1% in ASW between time points 21 and 60 days to finally increase to 13% at day 100 (Fig. 3B). The sharp drop in motility between time points 21 and 60 days in ASW coincides with the maximum *Synechococcus* cell densities (Fig. 1) and, hence, highest photosynthate production and availability. Very low levels of flagellin were also detected in previous studies where *R. pomeroyi* was grown in carbon-rich media (e.g., the flagellin was not detected in the exoproteome of this strain when grown in marine broth; Christie-Oleza and Armengaud, 2010; Christie-Oleza *et al.*, 2012b), highlighting the ability of *R. pomeroyi* to switch between a motile–nonmotile lifestyle, which is dependent on nutrient availability. In order to prove this hypothesis, we visualized *R. pomeroyi*'s motility under different nutrient conditions. No cell motility was observed when this strain was incubated in nutrient-rich marine broth (containing 0.6%, wt/vol, of organic carbon, of which 0.5% is peptone and 0.1% yeast extract) or in carbon depleted media (i.e., ASW or ASW supplemented with ammonium and vitamins). Interestingly, *R. pomeroyi* did show notorious motility when incubated in filter-sterilized ASW media obtained from a 2-week old *Synechococcus* culture (Supporting Information Movie S1), which we predict contained $\sim$ 0.02% (wt/vol) of organic carbon (i.e., mainly protein) based on previous measurements (Christie-Oleza *et al.*, 2017). We then tested the motility of *R. pomeroyi* in the presence of varying concentrations of four different sources of organic matter (Fig. 5 and Supporting Information Movies S2 and S3). Glucose, yeast extract and peptone induced cell motility at concentrations as low as 0.005% (wt/vol). In contrast, higher

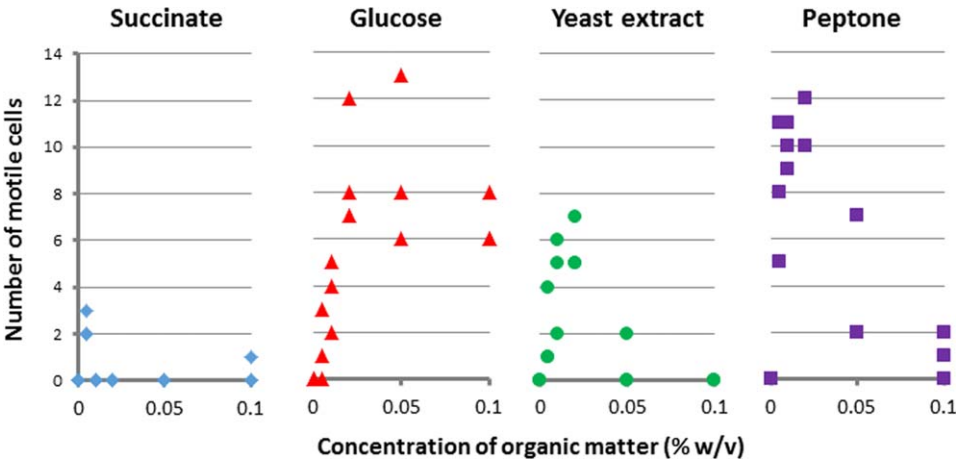

Fig. 5. Motility of *R. pomeroyi* DSS-3 in the presence of varying concentrations of different organic carbon sources. Quantification of motility was based on the number of moving cells observed in 10-s videos obtained from three different fields per condition.

substrate concentrations of yeast extract and peptone (i.e., 0.1%) caused a drop in motility (Fig. 5), suggesting a substrate repression in flagella biosynthesis as reported previously in other heterotrophic bacteria (Stella *et al.*, 2008). These results are in agreement with the proteomic detection of flagellin when *R. pomeroyi* was grown in the presence of *Synechococcus* and demonstrates an adaptive life strategy of this strain in response to different levels of organic matter. Hence, *R. pomeroyi* remains immotile in total absence of a carbon source and very low concentrations of organic matter are enough to switch on its motile phenotype. The drop in flagella abundance observed in natural SW incubations (Fig. 3) is possibly caused by the lower concentrations of organic carbon produced by the phototroph under such nutrient-deplete conditions. Previous measurements of organic matter in such oligotrophic conditions suggest concentrations as low as $\sim$ 0.0002% (wt/vol). While we did not test such low substrate concentrations in our controlled experiments (Fig. 5), these small amounts of substrate seem to be enough to induce a low level of flagella production and, hence, motility in *R. pomeroyi*. This is also supported by previous observations of 5.0–6.5% flagellin detected in an exoproteomic analysis where this heterotroph was incubated in natural coastal seawater (Christie-Oleza *et al* 2012b). The abrupt decrease in flagellin abundance observed in nutrient-enriched ASW conditions (Fig. 3) is more likely caused by elevated organic matter concentrations (i.e., $>$ 0.1%), which represses the biosynthesis of this structure. This may well simulate a phytoplankton bloom where the heterotroph turns off its motility in order to remain within the high concentration patch.

Membrane transport. This is the most abundant category of proteins detected in the exoproteomes of *R. pomeroyi* (42% and 38% in ASW and SW, respectively), mostly made up of outer membrane transport systems and the periplasmic components of inner membrane transporters. For example, the periplasmic substrate-binding component of ABC transporters made up 100% of the proteins detected for these transport systems, whereas the other components (i.e., permeases and ATP-binding proteins) remained undetected (Supporting Information Table S5). It is interesting to note that the abundance of membrane transporters mirrors the one of cell motility and highlights how the cell invests resources in response to nutrient availability (Fig. 3B). This generalist marine heterotroph is adapted to scavenge a large variety of scarce nutrients in oligotrophic marine systems and to proliferate rapidly in nutrient-rich patches of the ocean (Moran *et al.*, 2004; Newton *et al.*, 2010; Christie-Oleza *et al.*, 2012a,b). The data we present here suggest that this life strategy is achieved by splitting resources between motility to search for nutrients when these are scarce and to rapidly take them up through the investment in membrane transporters when they are abundant. As expected, *R. pomeroyi* induces the production of a large array of specific membrane transporters for importing amino acids, amines and carbohydrates in the presence of *Synechococcus*, which is not surprising as these are the main components of the cyanobacterium photosynthate (Christie-Oleza *et al.*, 2017). Nevertheless, while all transporters showed an increase in abundance in accordance to the pattern shown by the 'transport' category in Figure 3B, the periplasmic component of the manganese ABC transporter showed a decrease in abundance in both SW and ASW conditions (Fig. 6). Although it is not clear which specific metal is transported by this transporter (i.e., manganese and zinc), *R. pomeroyi* clearly decreases its uptake possibly favoring the phototroph's high demand in metals for primary production.

Interaction. The genetic transfer agent (GTA) and Repeats-in-ToXin (RTX-like) proteins are the main contributors of this functional category. While GTAs are bacteriophage-like particles produced by a large variety of

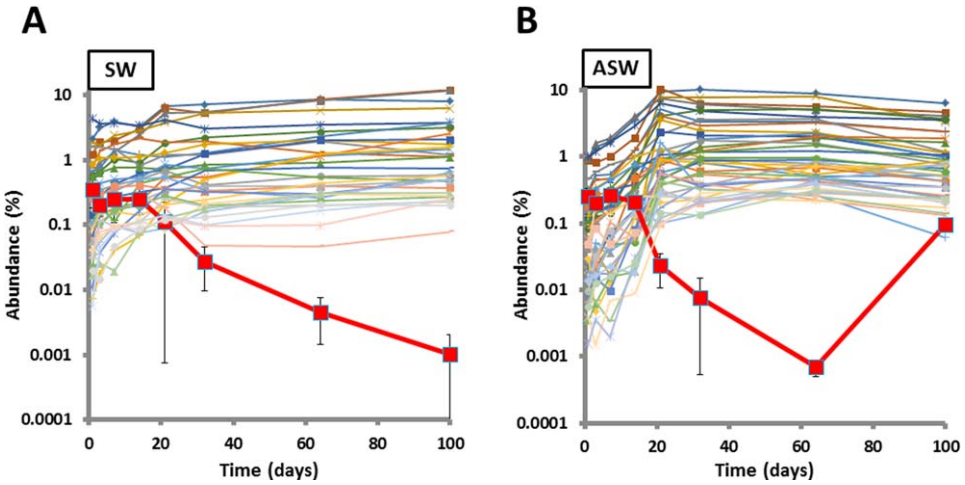

Fig. 6. Abundance of *R. pomeroyi* DSS-3 membrane transport proteins in natural SW (A) and ASW medium (B) over time. Only transporter proteins with average abundance above 0.1% are represented. The average value of triplicate cultures analyses ($n = 3$) are shown. The periplasmic component of the manganese ABC transporter is highlighted in bold and, for convenience, error bars showing standard deviation were added for only this protein.

bacteria which carry random fragments form the host's genome and that are thought to encourage horizontal gene transfer (Frost *et al*., 2005), RTX-like proteins are elements that mainly play a direct role in cell-to-cell interactions ranging from toxicity to adhesion, although due to the enormous extant variability of these proteins, most are of unknown function (Linhartova *et al*., 2010). Interestingly, while RTX-like proteins were highly produced in organic nutrient-rich broths, for example, the RTX-like protein PaxA represented over 50% of the proteins in the exoproteome when *R. pomeroyi* was grown in marine broth (Christie-Oleza and Armengaud, 2010), this does not occur in the mineral media used in this study, where the heterotroph is constantly supplied with a smaller amount of photosynthate. In any case, RTX-like proteins were still detected throughout the 100-day experiment (1.7% and 0.5% in ASW and SW, respectively). GTA are commonly found in *Roseobacter* strains (Biers *et al*., 2008; Zhao *et al*., 2009). The high production of GTA by *R. pomeroyi* when grown in co-culture with *Synechococcus* was highlighted previously (Christie-Oleza *et al*., 2015b). However, from the time course data, it is interesting to note that the abundance of GTA elements (with an average protein abundance of 4.6% and 5.7% in ASW and SW, respectively) mimics that of the flagella, that is, dropping from 5% to 9% during days 1–14, to between 0.1% and 0.3% during time points 21–64 days in ASW (Supporting Information Table S5). This suggest a co-regulation between the motile 'scavenging-for-nutrients' lifestyle mode and the burst of GTA production, possibly induced by the sensing of low nutrient availability. In fact, GTA production is known to be inhibited by high concentrations of phosphate (Westbye *et al*., 2013).

Hydrolytic enzymes for polymeric organic matter. One of the aims of this study was to highlight and monitor the production of potential secreted enzymes involved in the breakdown of phototrophic DOM over time. Marine Alphaproteobacteria are considered to have a very low extracellular hydrolytic potential due to the low number of enzymes that are encoded in their genomes (Barbeyron *et al*., 2016). In fact, previous exoproteomic analyses from a range of *Roseobacter* strains showed that different isolates produced a small but highly divergent, repertoire of exoenzymes suggesting that closely related species may be able to target different substrates and escape competition through the diversification of resources (Christie-Oleza *et al*., 2015b). Here, the overall average abundance of this category was 5.3% and 5.5% for ASW and SW, respectively, but while the abundance of these proteins progressively dropped in ASW (7.4% at day 1 to 4.1% at day 100), an opposite trend was observed in SW with an increase from 3.3% to 7.6% during the 100-day time course (Fig. 3B). Nineteen potential hydrolytic enzymes produced by *R. pomeroyi* were detected during this study (i.e., with an average detection $>$ 0.1% in at least one of the culture conditions; Table 2). The two most abundantly detected hydrolases were AAV93776 (2.1% and 2.7% in ASW and SW, respectively) and AAV95476 (0.9% and 2.68% in ASW and SW, respectively), re-annotated here as a possible pectate lyase and sialidase, respectively (Table 2). Hence, both proteins may be involved in polysaccharide hydrolysis, generating oligosaccharides and short chain sugars that can then be assimilated by the heterotroph. Despite being highly abundant, the pectate lyase-like protein shows a drastic reduction in the ASW co-culture between time points 21 and 60 coinciding with the period when *Synechococcus* is most abundant (Fig. 1). During this time period, *Synechococcus* is possibly at its maximum production of photosynthate and *R. pomeroyi* may fill its carbon and energy demands through the use of protein and other organic nitrogen compounds. Interestingly, it is between days 21 and 60 when *R. pomeroyi* shows an increased production of proteases

Table 2. Hydrolytic enzymes detected over time in the exoproteome of *R. pomeroyi* DSS-3 when co-cultured with *Synechococcus* sp. WH7803.

Locus ID	Annotation (possible function)	Substrate		Day 1[a]	Day 3[a]	Day 7[a]	Day 14[a]	Day 21[a]	Day 32[a]	Day 60[a]	Day 100[a]	Average
AAV93776	Hypothetical SPO0459 (pectate lyase)	Polysaccharides	ASW	4.94	4.05	3.23	3.08	0.04	0.02	0.08	1.63	2.13
			SW	1.43	1.88	2.16	2.29	3.08	3.12	3.84	3.61	2.68
AAV95476	BNR/Asp-box protein (sialidase)	Polysaccharides	ASW	1.14	1.00	0.99	1.35	0.80	0.43	0.51	1.07	0.91
			SW	1.07	1.08	1.31	1.22	1.77	1.59	1.49	1.25	1.35
AAV95139	Twin-arginine pathway (phosphatase PhoX)	Phosphates	ASW	0.58	0.56	0.40	0.33	0.16	0.88	0.65	0.15	0.46
			SW	0.53	0.54	0.57	0.28	0.13	0.08	0.02	< 0.01	0.27
AAV96145	Ser/Thr Phosphatase/nucleotidase (nucleotidase)	Nucleotides	ASW	0.04	0.05	0.27	0.13	0.93	0.77	0.70	0.34	0.40
			SW	0.06	0.18	0.23	0.44	0.69	0.58	1.02	1.49	0.59
AAV95890	Protease, S2 family (serine protease)	Proteins	ASW	0.03	0.01	0.01	0.02	0.51	0.56	0.55	0.09	0.22
			SW	< 0.01	0.01	0.01	0.01	0.01	0.03	0.07	0.13	0.03
AAV96272	Metallo-beta-lactamase (alkyl sulfatase)	Others	ASW	0.06	0.11	0.12	0.22	0.38	0.26	0.28	0.22	0.21
			SW	0.03	0.10	0.23	0.28	0.35	0.37	0.38	0.43	0.27
AAV93580	Alkaline phosphatase (phosphatase PhoD)	Phosphates	ASW	0.01	0.04	0.03	0.01	< 0.01	0.43	0.78	0.01	0.17
			SW	0.01	0.06	0.04	0.02	0.01	0.02	0.01	< 0.01	0.02
AAV95931	LysM domain/M23/M37 peptidase (peptidase family M23)	Proteins	ASW	0.12	0.28	0.19	0.15	0.11	0.18	0.11	0.10	0.16
			SW	0.05	0.08	0.07	0.06	0.02	0.02	0.01	0.06	0.05
AAV97448	Amidohydrolase protein (amidohydrolase)	Others	ASW	0.04	0.03	0.04	0.04	0.32	0.26	0.27	0.16	0.14
			SW	0.02	0.07	0.11	0.15	0.15	0.08	0.18	0.40	0.14
AAV95236	Beta-lactamase protein (carboxypeptidase)	Proteins	ASW	0.01	0.05	0.24	0.36	0.03	0.05	0.05	0.09	0.11
			SW	< 0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
AAV96841	Peptidoglycan-binding protein (glycoside hydrolase)	Peptidoglycan	ASW	0.06	0.13	0.07	0.11	0.06	0.10	0.12	0.04	0.09
			SW	0.02	0.09	0.07	0.06	0.03	0.01	0.01	0.03	0.04
AAV94622	Periplasmic serine protease (serine proteases)	Proteins	ASW	0.04	0.05	0.04	0.11	0.06	0.06	0.04	0.03	0.05
			SW	0.01	0.02	0.01	0.04	0.02	0.02	0.01	< 0.01	0.01
AAV95689	Fumarylacetoacetate hydrolase (aromatic hydrolase)	Others	ASW	0.02	0.02	0.05	0.04	0.09	0.07	0.06	0.03	0.05
			SW	0.02	0.03	0.03	0.03	0.02	0.03	0.06	0.05	0.03
AAV96493	Glycosyl hydrolase, family 25 (acetylmuramidase)	Peptidoglycan	ASW	0.08	0.10	0.10	< 0.01	< 0.01	< 0.01	< 0.01	0.01	0.04
			SW	0.03	< 0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	0.01
AAV94066	Cyclase family protein (aromatic hydrolase)	Others	ASW	< 0.01	< 0.01	< 0.01	< 0.01	0.07	0.07	0.06	0.04	0.03
			SW	< 0.01	< 0.01	0.01	0.01	0.01	0.02	0.04	0.07	0.02
AAV93452	Murein endopeptidase (murein endopeptidase)	Peptidoglycan	ASW	< 0.01	0.01	0.02	0.02	0.01	0.02	0.08	0.02	0.02
			SW	< 0.01	< 0.01	0.02	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.01
AAV95558	Hypothetical SPO2296 (lysozyme)	Peptidoglycan	ASW	0.02	< 0.01	0.01	0.01	0.01	0.01	0.04	0.03	0.02
			SW	0.02	< 0.01	< 0.01	< 0.01	< 0.01	0.02	< 0.01	< 0.01	0.01
AAV94260	Amidohydrolase protein (amidohydrolase)	Others	ASW	< 0.01	< 0.01	< 0.01	< 0.01	0.04	0.02	0.02	0.01	0.01
			SW	0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
AAV96597	Peptidase, M16 family (peptidase M16)	Proteins	ASW	< 0.01	< 0.01	< 0.01	< 0.01	0.03	0.03	0.02	< 0.01	0.01
			SW	< 0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

a. The values shown are the average of relative abundance obtained from three biological replicate cultures. The full data can be obtained from Supporting Information Table S5.

(i.e., AAV95890 and AAV97448) and amidohydrolases (AAV97448 and AAV94260). Members of the *Roseobacter* group are known to preferentially target small nitrogen-rich DOM (Bryson *et al*., 2016; Teira *et al*., 2017), and hence, it is not surprising that these organisms will specialize in using these compounds when they are present. While the abundance of organic matter in ASW allows *R. pomeroyi* to shift its targeted polymeric DOM, in SW it shows a more sTable production of hydrolases throughout the 100-day co-culture, with only the increase of the pectate lyase AAV93776 (from 1.4% to 3.6%) and the protease AAV95890 (from $<$ 0.01% to 0.13%) over time. *Ruegeria pomeroyi* encodes and produces most enzymes required to breakdown the polymeric components within the cyanobacterial photosynthate (i.e., protein, polysaccharides, peptidoglycan and other sulfur and amide compounds), and hence, it is able to mineralize most of the primary produced organic matter, enabling the establishment of long term co-cultures based on nutrient cycling described previously (Christie-Oleza *et al*., 2017). Nevertheless, we acknowledge that the identification of most hydrolytic enzymes in Table 2 requires further experimentation to confirm their predicted function. For example, the most abundant enzyme detected, i.e., the pectate lyase-like protein, was annotated as a hypothetical protein and only suggested here as a hydrolytic enzyme for polysaccharides based on a pectate lyase-like domain it contains. Other pectate lyases have been detected in marine microbes, but these seem to have been acquired from a terrestrial origin (Hehemann *et al*., 2017). Nevertheless, this is not the norm and most marine microbes have a novel array of untapped hydrolytic enzymes that largely differ from their well-known terrestrial counterparts (Hehemann *et al*., 2014), which require further characterization.

Hydrolytic enzymes for phosphate. Both alkaline phosphatases PhoX and PhoD encoded by *R. pomeroyi* were found in the exoproteomes (Table 2). PhoX, and not PhoA, is prevalent in marine microbes and is thought to play an important role in marine oligotrophic systems (Sebastian and Ammerman, 2009; 2011). PhoX was the predominant alkaline phosphatase in our dataset (Table 2). Nevertheless, the less-known PhoD, which was in very low abundance throughout most of the time course (abundance below 0.06%), peaked only at time points 32 and 60 (0.43% and 0.78%, respectively) under ASW conditions. While the substrates targeted by PhoX have been characterized (Sebastian and Ammerman, 2011), organic phosphate compounds targeted by PhoD remain unknown. *Ruegeria pomeroyi* also abundantly secretes the nucleotidase AAV96145 when grown in co-culture with *Synechococcus*. It is worth noting the progressive increase in this nucleotidase especially under SW conditions (from 0.06% to 1.49%), which coincides with a strong decrease in PhoX abundance (Table 2). The ABC transporters for phosphate and organic phosphate (e.g., glycerol-3-phosphate) remain constant during the whole time course (Supporting Information Table S5), suggesting that *R. pomeroyi*'s phosphorous starvation status does not vary over time, but the switch from PhoX to a nucleotidase indicates a variation in the organic phosphorous produced by *Synechococcus*. We hypothesize that *Synechococcus* may replace its phospholipids for sulfolipids (Van Mooy *et al*., 2009) and even initiate the accumulation of polyphosphates (Martin *et al*., 2014), which may become, together with nucleic acids, the main source of phosphorous in the system.

Conclusions

Microbial exoproteomes are good proxies to study the dynamic interactions of microbes with their environment. Here we generated a unique time course exoproteomic dataset of a 100-day long *Synechococcus* sp. WH7803–*R. pomeroyi* DSS-3 co-culture incubated in both nutrient rich and natural oligotrophic seawater. The observed protein variations matched well with the culture's physiology over time and emphasizes the need of time course experiments such as the one we present here in order to obtain a comprehensive understanding of microbial interactions, as single time points may be misleading. This study has highlighted a number of interesting aspects in this phototroph–heterotroph system such as (i) the heterotroph's varying motility lifestyle depending on nutrient availability, (ii) the unexplained selective leakage of phycobilisomes to the milieu by *Synechococcus*, (iii) the specificity of nutrient acquisition though the production of an array of active membrane transport systems, (iv) the large production of SOD by *Synechococcus* to deal with ROS despite the presence of a heterotroph, (v) the varying abundance of a type IV pili structure produced by the phototroph, (vi) a list of uncharacterized hydrolytic enzymes secreted by *R. pomeroyi* to mineralise the polymeric organic matter generated by the cyanobacterium, (vii) a pattern of phosphatases that varies over time and that is believed to adapt to the pool of organic phosphorous present in the system and (viii) a list of relevant proteins of unknown function that require further research. Hence, the high-resolution time course dataset we present here will become a reference for future characterization of specific molecular mechanisms involved in sustaining this microbial system.

Experimental procedures

Bacterial growth and experimental setup

Marine *Synechococcus* sp. WH7803 was grown in ASW (Wilson *et al*., 1996) at 22°C at a light intensity of 10 μmol photons m^{-2} s^{-1} with shaking (140 r.p.m.). *Roseobacter* strain *R. pomeroyi* DSS-3 was grown in marine broth (Difco,

France) at 28°C until stationary phase. *Ruegeria pomeroyi* and *Synechococcus* cells were harvested via centrifugation and washed twice in filter-sterilized autoclaved seawater (SW, natural seawater collected from the Gulf Stream in the Gulf of Mexico; provided by Sigma, USA) prior to co-inoculating both organisms in 100 ml ASW and SW at cell concentrations of $\sim 10^8$ and 10^7 cell ml^{-1}, respectively (Fig. 1). ASW and SW co-cultures were incubated for up to 100 days in optimal conditions for *Synechococcus* (as described earlier). We allowed triplicate flasks for each one of the eight time points analyzed, i.e., days 1, 3, 7, 14, 21, 32, 60 and 100. At each time point, *Synechococcus* cell abundance was monitored by flow cytometry (BD FACScan), while viable heterotrophs were counted by colony forming units on marine agar (Difco, France) as previously suggested (Christie-Oleza *et al*., 2017).

For motility visualization, *R. pomeroyi* was grown in 10 ml of marine broth for 40 h after which cells were washed in filter-sterilized autoclaved SW, re-suspended in 10 ml of SW and further incubated for 4 days to starve the cells. Then, 100 μl of starved cells were added to 100 μl of ASW supplemented with 2.5 mM of $(NH_4)_2SO_4$ and 4× the standard concentration of f2-media vitamin mix. Succinate, glucose, yeast extract (Merck, Germany) and bacto peptone (Merck, Germany) were added at final concentrations, 0.005%, 0.01%, 0.02%, 0.05% and 0.1% (wt/vol), and incubated for 4 h before imaging cell motility using concave microscope slides under a 100× objective of a light microscope (Nikon Eclipse Ti) equipped with a widefield camera (Andor Zyla sCMOS). For each one of the conditions, 10 s videos were recorded from three different fields containing approximately 60 ± 10 cells and the number of motile cells was counted.

Preparation of exoproteome samples

Triplicate 100 ml ASW and SW cultures were used for each time point. The exoproteomes contained in the culture milieu were collected after removing all cells via centrifugation at 4000 r.p.m. for 15 min at room temperature and further filtering the supernatant through 0.22 μm pore size filters (Millex-GV; Millipore, Germany). A total of 40 and 80 ml of the supernatant of SW and ASW cultures, respectively, were used for trichloroacetic acid precipitation as described previously (Christie-Oleza and Armengaud, 2010). The resulting protein pellets were dissolved in LDS loading buffer (Invitrogen, USA), and the equivalent of 20 ml of ASW cultures and 40 ml of SW cultures were loaded on a precast Tris-Bis NuPAGE gel (Invitrogen, USA) using 1× MOPS solution (Invitrogen, USA) as the running buffer. SDS-PAGE was performed for a short gel migration (5 mm). This allowed removing contaminants and purifying the polypeptides in the polyacrylamide gel.

Trypsin in-gel proteolysis and nanoLC-MS/MS analysis

Polyacrylamide gel bands containing the exoproteome were excised and standard in-gel reduction with dithiothreitol and alkylation with iodoacetamide were performed prior to trypsin (Roche, Switzerland) proteolysis (Christie-Oleza and Armengaud, 2010). The resulting tryptic peptide mixture was extracted using 5% formic acid in 25% acetonitrile and concentrated at 40°C in a speed-vac. For mass spectrometry, the samples were resuspended in 2.5% acetonitrile containing 0.05% trifluoroacetic acid and filtered using a 0.22 μm cellulose acetate spin column 16,000*g* for 5 min in order to eliminate undissolved aggregates. Sample were analyzed by means of nanoLC-ESI-MS/MS using an Ultimate 3000 LC system (Dionex-LC Packings) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, USA) using a 60 min LC separation on a 25 cm column and settings as previously described (Christie-Oleza *et al*., 2015b).

Data analysis

Raw files were processed using the software package for shotgun proteomics MaxQuant version 1.5.5.1 (Cox and Mann, 2008) to identify and quantify protein using the UniProt databases of *Synechococcus* sp. WH7803 and *R. pomeroyi* DSS-3. Samples were matched between runs. Other parameters were set by default. The peak intensities across the whole set of measurements were compared to obtain quantitative data for all the peptides in all the samples. Peak intensities and spectral counts assigned to each organism present in the co-culture were compared (Supporting Information Table S1). The list of detected peptides and polypeptides is provided as Supporting Information Tables S2 and S3, respectively. The bioinformatic analysis pipeline was completed using the software Perseus version 1.5.5.3. Decoy and contaminants were removed. The relative protein abundance was obtained from the raw protein intensities from each sample after normalization to protein size and prior to converting to a logarithmic scale with base 2 (Murugaiyan *et al*., 2016). The missing values were imputed using the default parameters. The protein quantification and calculation of statistical significance were carried out using two-sample Student's *t* test ($P = 0.05$) using a permutation-based false discovery rate ($q = 0.05$). Protein categorization was based on KEGG annotations with manual curation using the Conserved Domain search tool from NCBI. Prediction of secreted proteins was carried out using the servers SignalP 4.1 (Petersen *et al*., 2011), SecretomeP 2.0 (Bendtsen *et al*., 2005), LipoP 1.1 (Juncker *et al*., 2003) and PSORTb (Yu *et al*., 2010).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Spectral counts and mass intensities assigned to *Synechococcus* sp. WH7803 and *R. pomeroyi* DSS-3 in each one of the mass spectrometry runs. Percentage assigned to each organism in the co-culture is in brackets.

Table S2. List of peptides in the exoproteomes of *Synechococcus* sp. WH7803 and *R. pomeroyi* DSS-3 co-cultures detected by LC-MS/MS.

Table S3. Raw list of polypeptides detected by LC–MS/MS in the exoproteomes of *Synechococcus* sp. WH7803 and *R. pomeroyi* DSS-3 co-cultures.

Table S4. (a) Protein categories and comparative exoproteomic analysis of *Synechococcus* sp. WH7803 proteins detected in SW co-cultures with *R. pomeroyi* DSS-3. (b) Protein categories and comparative exoproteomic analysis of *Synechococcus* sp. WH7803 proteins detected in ASW co-cultures with *R. pomeroyi* DSS-3.

Table S5. (a) Protein categories and comparative proteomic analysis of *R. pomeroyi* DSS-3 proteins detected in SW co-cultures with *Synechococcus* sp. WH7803. (b) Protein categories and comparative proteomic analysis of *R. pomeroyi* DSS-3 proteins detected in ASW co-cultures with *Synechococcus* sp. WH7803.

Movie S1. *R. pomeroyi* incubated in filter-sterilized ASW obtained from a two-week old *Synechococcus* culture.

Movie S2. *R. pomeroyi* incubated in ASW supplemented with ammonium and vitamins. Only Brownian motion is observed.

Movie S3. *R. pomeroyi* incubated in ASW supplemented with ammonium, vitamins and 0.02% peptone (wt/vol).